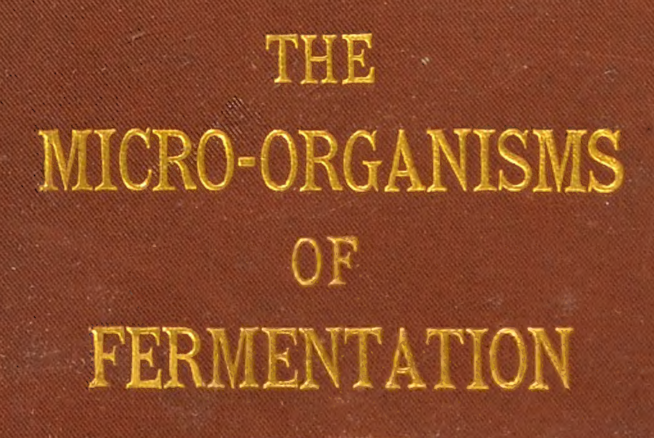
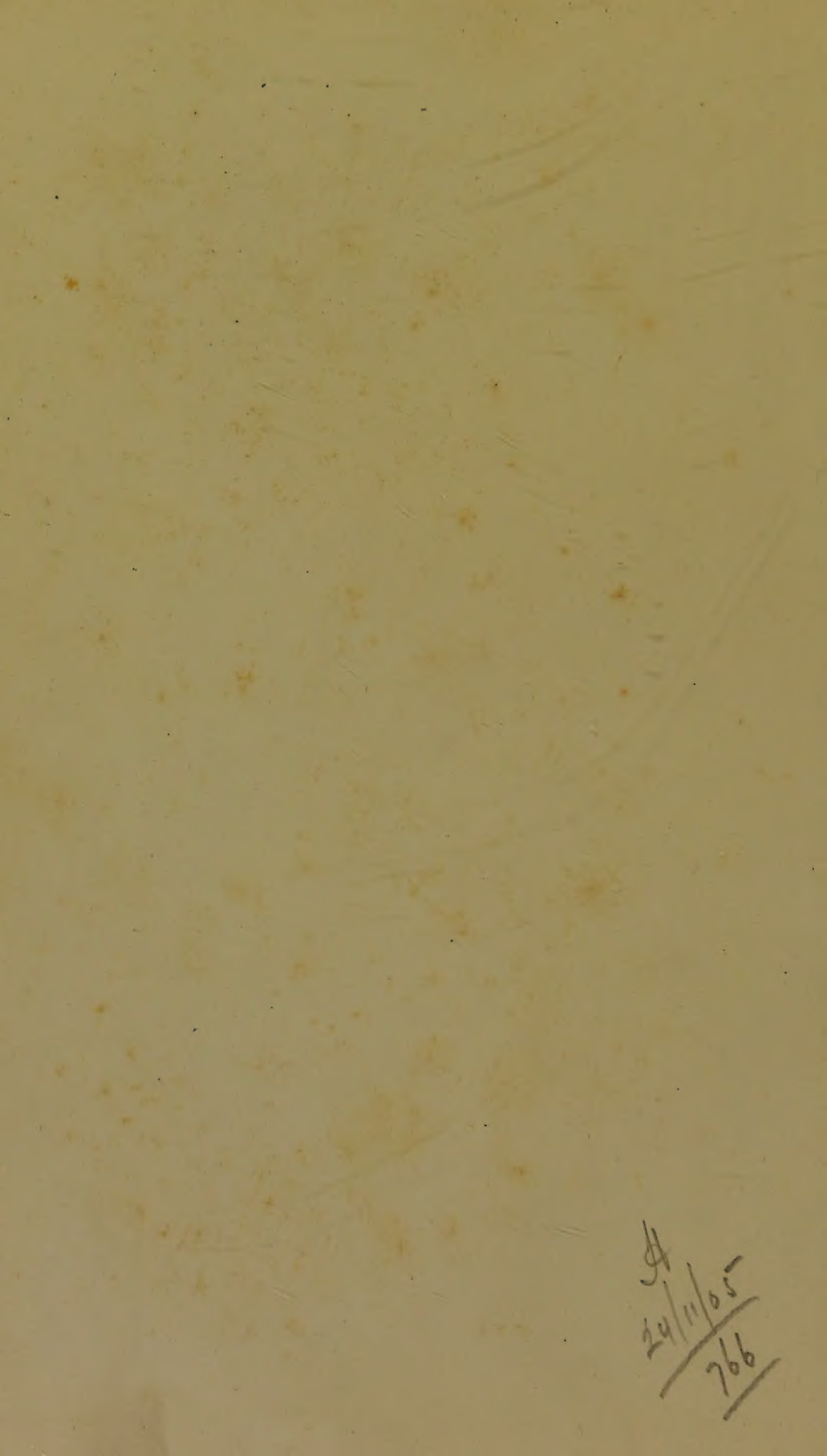


ALFRED JÖRGENSEN

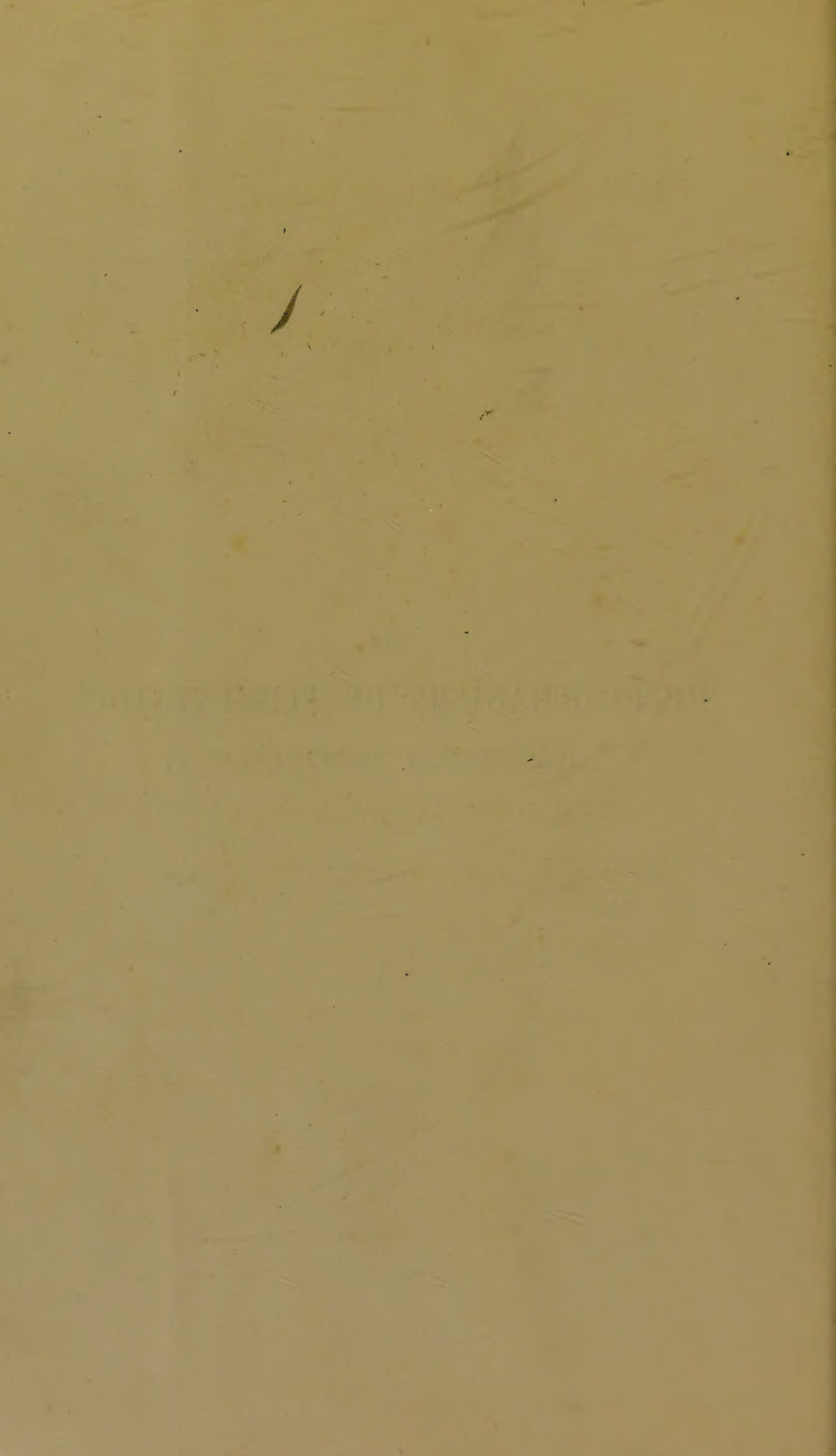




THE

MICRO-ORGANISMS OF FERMENTATION

PRACTICALLY CONSIDERED.



THE MICRO-ORGANISMS

OF

FERMENTATION

PRACTICALLY CONSIDERED.

By ALFRED JÖRGENSEN.

Edited from the German

By G. HARRIS MORRIS, Ph.D., F.C.S., F.I.C., etc.

Revised and Annotated by the Author and Editor,

WITH AN INTRODUCTION

By HORACE T. BROWN, F.C.S., F.I.C., etc.

With Thirty-six Original and Three Additional Illustrations.

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1889.

PREFACE TO THE ENGLISH EDITION BY THE AUTHOR.

DURING the three years which have elapsed since the first German edition of this book was published, the subject matter has received important additions in two directions, namely—

1. From a large series of scientific investigations by Hansen and other workers; researches which have been of the greatest importance in adding to our knowledge of the physiology and morphology of the micro-organisms of fermentation.

2. From a great number of communications from different beer-producing countries, which have rendered it evident that Hansen's system is of the greatest practical and industrial value.

These researches have made it possible to show, in a clearer and more exact manner than in the German edition, the central and fundamental theme of this book, namely, that the great progress made in our time in the physiology and morphology of the micro-organisms of fermentation and in the fermentative industries was initiated by Hansen when he took up the study of the question from a different point of view from that of his great predecessor, Pasteur. For this reason these remarkable investigations form the main part of the book.

It has been proved in a practical manner that the application of Hansen's discoveries to bottom-fermentation breweries

was an essential advance. It has also been proved in several breweries in different countries that a pure culture of selected high-yeast brings with it a greater security, and therefore possesses greater advantages for industrial purposes than ordinary impure yeast. The final solution of this question with respect to English breweries must, of course, also depend on the results of practical experiments; and, in concluding this preface, I must express the wish that the English brewer may shortly derive the same benefit from the investigations of my countryman as has been the case for years with his Continental *confrère.*

ALFRED JÖRGENSEN.

May 31*st*, 1889.

ERRATA.

Page 48, line 26 from top, insert *not* after *is*.
,, 138, ,, 20 ,, ,, for *probably*, read *of course*.

PREFACE BY THE AUTHOR.

As the owner of a laboratory devoted to the technology of fermentation, whose object, in addition to performing the analyses connected with this branch of applied science, consists in submitting to investigation the micro-organisms which play a part in this industry, I felt the want of a short handbook, which, amongst other purposes, might be used as a guide to this question.

The present work is an attempt to supply this want, and my aim has also been to provide a supplement to the text-books generally known and used in the different branches of the fermentation industries. Among these works I may especially mention Lintner's "Lehrbuch der Bierbrauerei" and Maercker's "Handbuch der Spiritusfabrikation" as typical examples of a good text-book. From the nature of the thing, it is necessary that these books should be written chiefly from the chemical standpoint, since the historical development has until now placed Chemistry in the front rank among the scientific handmaids of the industry. During the last ten years a new field for investigation—the botanical-physiological—has been opened up, and it has already yielded important practical results. The literature connected with this, and the work which is necessary in order to obtain a grasp of this new line of investigation, are already so comprehensive that a division of labour appears necessary, and the authors of the above-mentioned text-books cannot be blamed for continually assigning a somewhat secondary position to this branch of the subject.

I have further endeavoured to write a popular book in the true sense of the word, with the special limitation of selecting

and mentioning from the vast literature which already exists, only that which is necessary for the knowledge of the most important industrial organisms. The multifarious theories and hypotheses will therefore be only noticed here, when by their criticism a useful lesson—which unfortunately is often purchased dearly by many practical men—can be conveyed.

The introductory remarks on the scientific methods of investigation should be grasped—partly as a preliminary guide to the researches of the physiology of fermentation, and partly as statements, the comprehension of which must be the conditions for the correct understanding of the more special contents of the book.

As is known, books at present exist in which the most prominent literature on micro-organisms is collected. My work chiefly differs from these in—

(1) That it treats not only of the bacteria, but also of the mould- and yeast-fungi; and

(2) That it, as before stated, pays exclusive attention to the fermentation industries.

The most recent literature has been employed in the preparation of the book, and naturally special attention has been paid to the work which has cleared the road in many directions, and for which we have to thank Dr. E. C. Hansen, of the Carlsberg Laboratory. Among the manifold communications which Hansen has made to me in the course of the year there have been some observations which, with his permission, are here published for the first time. I have to express my heartiest thanks to him for this.

ALFRED JÖRGENSEN.

COPENHAGEN, *March*, 1886.

PREFACE TO THE ENGLISH EDITION.

THE Introduction which Mr. Horace T. Brown has so kindly contributed to this edition renders it unnecessary for more to be said here than that this translation has had the advantage of being revised by the Author, and many additions have also been made both by him and by myself, thus bringing the work well up to date; whilst I have added an Appendix, with illustrations, on the pure culture of yeast on an industrial scale, and an Index, both of which were wanting in the German edition.

The indulgence of the reader is asked for the many faults of diction and grammatical construction which I am only too conscious exist in this edition; such faults are, however, more or less unavoidable in a translation if the original text is at all adhered to.

G. H. M.

BURTON-ON-TRENT, *March*, 1889.



INTRODUCTION.

The publication of "Die Micro-organismen der Gärungs-industrie," by Alfred Jörgensen, in 1886, for the first time placed before the technologist, in a concise and readable form, a complete account of the numerous researches upon the micro-organisms which play such an important part in the wine, beer, and distillation industries of the world. The work in question undoubtedly supplied a long-felt want in technological literature; and when my colleague, Dr. G. H. Morris, consulted me as to the desirability of producing an English translation of the book, I agreed with him that by so doing he would be conferring a benefit upon a large and rapidly increasing number of technologists in this country, who are ever anxious to keep abreast of all scientific investigation which has any bearing upon the industries in which they are engaged.

It is no exaggeration to say that the classical researches of Pasteur upon the alcoholic and lactic fermentations, published 1857–61, and followed by a critical study of the fermentative processes involved in the manufacture of wine, vinegar, and beer, really lie at the root of all our present knowledge of those micro-organisms which stand in causal relation to fermentation, putrefaction, and disease. The importance now attached to such studies may be measured by the ever-increasing accumulation, in our scientific and medical journals, of communications bearing upon the morphology, life history, and functions of these minute organisms, which we now recognize as playing such an important part in Nature's economy. Their study has been, of late, elevated into a special branch of Biology, which is now very generally known as Bacteriology. This term, first

introduced by pathologists, who seldom have to deal with any of the micro-organisms which may not be classed with the Bacteria, is an objectionable one in several respects more especially as, strictly speaking, it excludes organisms such as the Saccharomycetes, the accurate study of whose life-history and functions has been really the starting-point and foundation of this very branch of science.

The special treatises upon Bacteria and their allied forms, which have appeared within the last few years, have treated of these organisms principally in their pathogenic aspect, and have, I think, laid too little stress upon the debt which the pathologist owes to the fermentation industries generally; yet it is abundantly evident that the principles first laid down in the "Études sur le Vin," the "Études sur le Vinaigre," and the "Études sur la Bière," rendered it possible for Koch to establish, on the one hand, the causal relation of the *Bacillus anthracis* to splenic fever, and on the other, the similar relation of the *Bacillus tuberculosis* to tubercular disease.

It is now but little more than a quarter of a century since Pasteur established on a firm basis the vitalistic theory of fermentation, and we know that he did not relinquish the study of the industrial processes which had afforded him such ample means of establishing his theory until the year 1876. A little prior to this time the knowledge, slowly but surely acquired, that all fermentative and putrefactive changes are the concomitants and the physiological expression of minute organisms, had begun to make itself felt in the domain of experimental physiology, and in the etiology of the infectious diseases had given new life and vigour to the theory of "living contagia," a theory which had been arrived at deductively, and very clearly expressed, as far back as 1840, by Henle. In Pasteur's earlier works we often find indications of a belief that his labours would ultimately have an important bearing upon the contagious diseases, but with characteristic caution, when convincing experimental evidence is wanting, we find this opinion expressed more as an article of personal faith than as compelling the judgment

of the reader. As late as the year 1876, for instance, in the preface to his "Études sur la Bière," the last of his investigations of a purely industrial character, we find the following passage :—"Ces nouvelles études reposent sur les mêmes principes qui ont servi de guide à mes recherches sur le vin, le vinaigre, et la maladie des vers à soie, principes dont la fécondité et les applications sont, à mon avis, sans limites. L'étiologie des maladies contagieuses est peut-être à la veille d'en recevoir une lumière inattendue." At the time when this passage was penned Robert Koch had already commenced those investigations which have shed such a lustre upon his name; and we know that Pasteur himself, pursuing the logical course marked out in the words quoted above, began, henceforth, to devote his splendid genius to the elucidation of problems connected exclusively with the etiology of disease.

Within the period embraced from 1870 to 1876, the study of the micro-organisms divided into two distinct branches, and the two streams of investigation, the biological and the industrial, henceforth continued their course more or less independently of each other. It is the more direct line of current, the study of the fermentation-organisms properly so-called, which most concerns us at the present moment.

The progress of investigation in this direction has been influenced in a very marked degree by the researches emanating from the Carlsberg laboratory. This institution, founded and endowed in 1875, by the well-known Danish brewer, the late J. C. Jacobsen, had for its object, in the words of the founder, "de vérifier par des recherches originales les doctrines déjà etablies par la science, et de les développer par des études suivies, de manière à en former une base scientifique aussi complète que possible pour les opérations du maltage, du brassage, et de la fermentation."

The numerous papers of high scientific merit which have appeared from time to time in the "Meddelelser fra Carlsberg Laboratoriet," the official publication of the Institute, show clearly how well the intentions of its founder have been carried out, and his hopes realized. In the volume for 1879

we find, under the head of "Contributions à la connaissance des organismes qui peuvent se trouver dans la bière et le moût de bière, et y vivre," the first of a series of remarkable papers by Dr. Emil Chr. Hansen. These investigations may justly be said to have inaugurated a new era in the study of the Saccharomyces and their allied organisms.

Admirably prepared and disciplined for such studies, Hansen brought to bear upon them the trained mind and eye of the cryptogamic botanist, and soon struck out new and original lines of research which have led to results of the highest scientific and practical value.

One great practical outcome of Hansen's investigations has been to establish, contrary to the opinion of most observers before him, that the micro-organisms which exert a prejudicial effect upon our brewing processes are by no means confined to the class of the *Schizomycetes*, but that several species and varieties of the *Saccharomycetes* are capable, under certain conditions, of exerting a very unfavourable influence, especially those so-called "wild" forms whose natural habitat is the surface of ripe fruits and grain, and which are found abundantly in the air at certain periods of the year. He has moreover shown that the supposed morphological differences which have been relied upon to establish the various species and varieties of the Saccharomyces, are in the highest degree illusory and untrustworthy; in fact, that species, having very different physiological functions, may assume forms which it is impossible to distinguish by a mere microscopical examination. The methods of analysis employed by Hansen for the detection of the "wild" species in a yeast which, under the microscope, apparently consists of but one form, are fully described in Jörgensen's work, and they merit our closest attention. It is, however, in the perfection of the methods of pure yeast culture that Hansen has perhaps made his greatest and most enduring mark upon the brewing industry. The present work contains an admirable account of these beautiful processes, by the aid of which it is now possible to cultivate, for manufacturing processes, a stock of yeast whose unmixed nature is ensured by the fact that it

has been propagated from a *single yeast cell*, previously selected and marked under the microscope.

The application of Hansen's methods of pure yeast culture to the every-day requirements of the brewer is rapidly gaining ground on the Continent, but for various reasons it has made little or no progress in this country. This is not altogether due, as some imagine, to the apathy and conservatism of our English brewers, but to the particular conditions under which the high fermentation brewer has to carry on his processes, and to the entirely different requirements of our beer as regards secondary fermentation, and after-treatment in store.

After a considerable amount of experience in the employment of pure yeast culture on an industrial scale, my colleague and I are convinced that many difficulties have yet to be surmounted before the English brewer can rely upon it with as great a confidence as can his Continental *confrère*.

I am, nevertheless, confident that, in a more or less modified form, pure yeast culture will play a very important part in the brewing of the future in this country. From a mere laboratory stand-point the problem is a very simple one, but unfortunately it is one which cannot be solved entirely in the laboratory. It is only by working on a large scale in the brewery itself that we can properly appreciate those minute differences of function which mark the closely allied varieties of the Saccharomyces, and which, small as they are, have so marked an influence upon the flavour and general properties of the product. I can only hope that the appearance of Jörgensen's "Micro-organismen der Gärungs-industrie," in an English dress, may encourage many of our brewers to enter upon the long-continued, careful, and patient study of these questions which is still necessary before we can adopt with confidence this new application of exact scientific methods to an important industry.

HORACE T. BROWN.

BURTON-ON-TRENT,
February 12th, 1889.

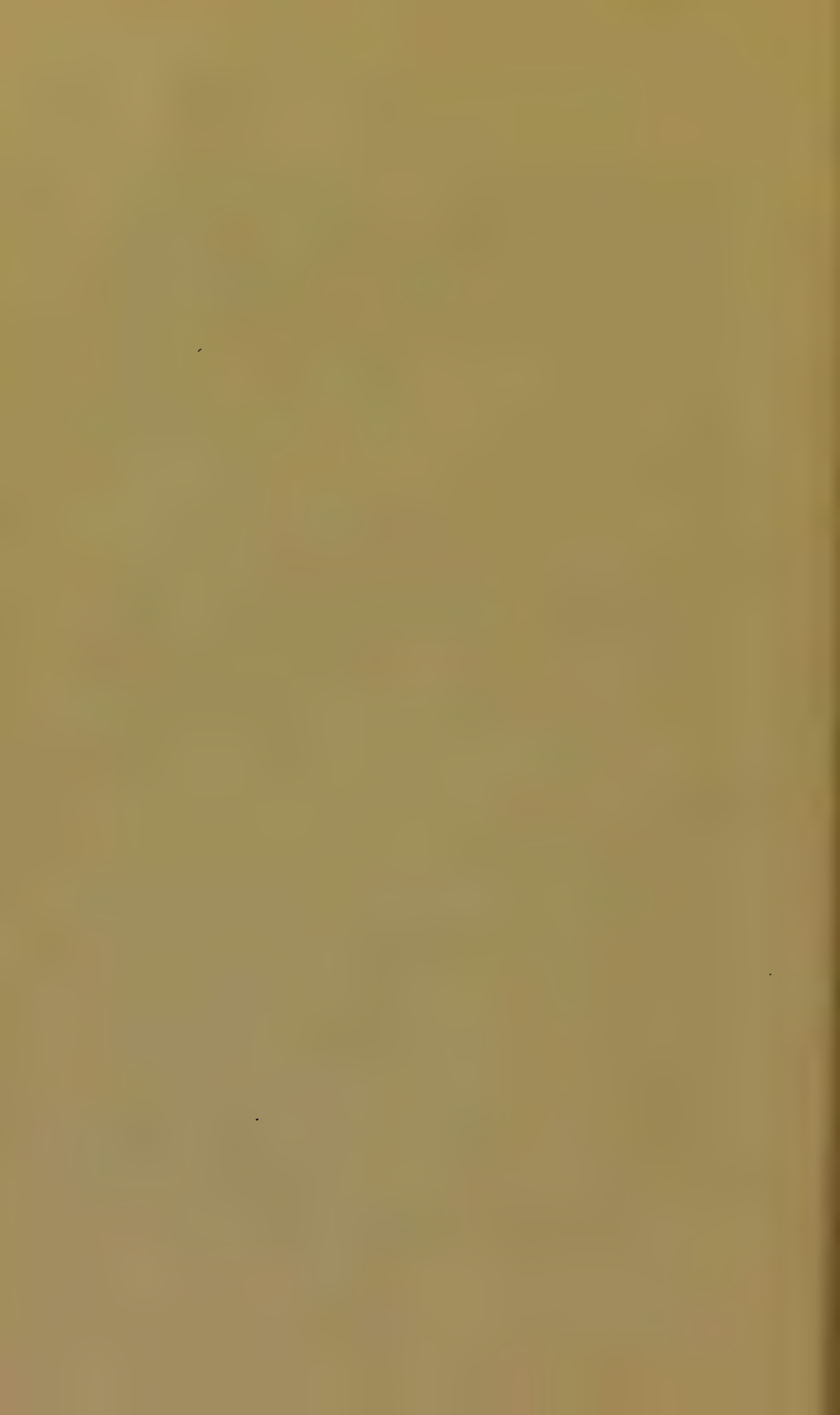


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CHAPTER I.

MICROSCOPICAL AND PHYSIOLOGICAL EXAMINATION.

THE microscope will be for all time the most important aid in the investigation of micro-organisms, since these, as individuals, are always undistinguishable by the naked eye. The earliest observations of any great importance in the physiology of fermentation we owe to purely microscopical investigations, and we must go back far beyond the middle of the present century in order to see the commencement of biological and physiological investigations. After a certain probability had arisen that the same species of micro-organism did not always occur in the same form, there began an active work in various directions with the so-called "culture experiments," in which endeavours were made, by artificially varying the conditions of growth, to study the various stages of development of the same species in order to determine its entire life-history. The idea was correct, but the performance was, however, at that time so faulty that "culture experiments" threatened in consequence to fall into complete discredit. The experiments were performed without necessary precautions being taken, as the following example shows:—Beer yeast was sown on a moist piece

of bread, the cultivation was carefully covered with a glass shade, and all possible precautions were observed in order to protect the growth from external contamination. After some days a growth of mould appeared, as is always the case when moist bread is placed under these conditions; and the conclusion was therefore drawn that beer-yeast was the origin of the mould forms, and that, consequently, yeast- and mould-fungi were different phases of growth of the same species.

A number of years elapsed before what now appear to be the most self-evident requirements of this class of investigations were put in practice, before it was possible to be certain from which point to start in order to arrive at a definite conclusion. These requirements were gradually practised with greater care, and at last, as we shall see later, a point was reached which satisfies in the highest degree both the demands of our subject and also those of the closely related branches of science.

A microscope capable of giving a magnification of 1,000 diameters is, as a rule, necessary for the investigation of micro-organisms. For the yeast- and mould-fungi the only preparation generally required consists in placing a drop of the liquid containing the organism on a glass slide, and spreading it out in a thin layer by means of a cover-glass. When cultivated on solid substances, a very small portion of the growth is first mixed with a drop of water. The preliminary examination of bacteria also must always be carried out in the above manner. In the latest investigations with bacteria, and especially with the pathogenic forms, a number of different methods of drying and staining are employed, partly in order to facilitate the examination, and partly in order to bring out characteristics which would otherwise be observed only with difficulty or not at all. An objection to these methods, urged with undoubted correctness, is that the violent treatment often alters certain proportions of the bacteria; for instance, the proportion of length and thickness. On the other hand, it must be admitted, especially with regard to certain pathological forms—the tubercle-

bacillus, for instance,—that in consequence of Koch's examination of such preparations it first became possible to determine the organism with certainty; and, indeed, staining is often necessary in order to detect this bacillus. As an example of the methods of staining, we will go somewhat more closely into the examination of the tubercle-bacillus, which is one of the most important observations made in modern medicine. Koch gives the following method for its examination:—The section of the tissue which contains the bacilli is immersed for twenty-four hours in a mixture of 100 parts of distilled water, 1 part of concentrated alcoholic solution of methylene blue, and 0·2 part of a 10 per cent. potash solution. By this treatment the section is stained dark blue; it is then immersed in a concentrated aqueous solution of vesuvin for fifteen minutes. The section is now rinsed in distilled water until the blue colour disappears and a more or less strong blue tincture remains behind; finally, it is treated with alcohol, mounted in clove oil, and examined. The cell-nuclei and most species of micrococci are thus stained brown, the tubercle-bacilli an intense blue. (Of the known species of bacilli, only the bacillus of leprosy behaves in the same way, and this is easily distinguished by other means from the bacillus of tuberculosis.) In Koch's method it is necessary that the staining solution should be alkaline, since this bacillus does not take the stain in acid or neutral solutions; the neutral solution of another colouring matter removes the first stain in all cases with the exception of that of the tubercle-bacillus, which retains the original staining. Later there were many other methods proposed for the identification of this micro-organism, the most preferable of which is that of Ehrlich. This investigator used aniline instead of potash. Aniline is a slightly yellow, oily liquid, the saturated aqueous solution of which takes up more colouring matter than that of potash. He has also employed mineral acid for decolourizing, proceeding on the supposition that the tubercle-bacillus is surrounded by a cell-wall which is only permeable by alkaline liquids. Therefore, when the alkaline solution of the colouring matter stains the bacilli, cell-nuclei, membrane, &c., and the first-named are

difficult to find in the mixture, treatment with an acid removes the stain from all parts of the section and from all foreign organisms, and, since the presumptive cell-wall of the tubercle-bacillus is not penetrated by acid, these bacilli remain as the only stained bodies in the otherwise decolourized material. Ehrlich carries out the staining in the following manner :—Finely powdered gentiana-violet is dissolved in a saturated aqueous solution of aniline ; 10 to 12 drops of this solution are filtered into a watch-glass, and the sections to be examined are allowed to remain in this for twenty-four hours. They are then rinsed with distilled water, and again placed in a watch-glass with a solution of 3 parts of nitric acid to 100 parts of alcohol. After three to five minutes the sections are decolourized; they are then transferred to pure alcohol, and finally examined in clove oil.

As is known, photographic illustrations of bacteria have recently come into use very much, having been first introduced by R. Koch. In order to obtain these, staining and decolourization are necessary, partly in order to render the contour of the bacteria sharper, and partly in order to remove all bodies detrimental to the picture.

Staining and decolourization are seldom necessary for examinations connected with the physiology of fermentation, where the organisms are always free and only seldom mixed with disturbing elements, and only in a few cases does staining indicate characteristics of species (*Mycoderma aceti* and *Myc. Pasteurianum*).

It is, however, sometimes necessary in investigations with the organisms of fermentation, and especially with bacteria, to adopt another method of preparation. The secretions of organic and inorganic nature which appear in the solutions have often a deceptive similarity to different bacterial forms, and it is then only with the greatest difficulty, and, indeed, sometimes even impossible, for the most experienced observer to determine with certainty whether the small spherical bodies in the field of the microscope are spherical bacteria or secretions from the solution. In such doubtful cases it is advisable, before entering on the physiological examinations

later to be described, to have recourse to micro-chemical reagents, which often give good preliminary hints. In beer and in nutritive liquids generally which contain albuminoids, these often separate in spherical and thread-like forms; the starch granules, the dextrins formed from starch, and even some of the hop constituents may also appear as small spherical bodies. The addition of a small quantity of alcohol, ether, chloroform, acetic acid, caustic soda, or potash solution, &c., is often able to throw some light upon the nature of these bodies, since the resinous substances are soluble in the former liquids, and the amorphous albuminoid matter more or less soluble in the latter solutions; the addition of iodine will colour blue the starch granules which are present, whilst certain dextrins are coloured red by iodine.

With the higher organisms of fermentation—yeast- and mould-fungi—staining is employed for other purposes, namely, in order to obtain information concerning the substances which are found in the cell-wall or cell-contents at different stages of their growth. On the addition, for instance, of a solution of iron chloride or any other salt of iron to cells which contain tannic acid, a bluish-black or green coloration is formed in the cells; it was observed in this way that the cells of *Saccharomyces cerevisiæ* contain a considerable quantity of tannic acid during the earlier stages of fermentation. If yeast cells are treated with a solution of hæmatoxylin or osmic acid, small, sharply defined, dark-coloured bodies are seen, which may be considered to be cell-nuclei of the same nature as those generally observed in the cells of the greater number of plants without the aid of this treatment.

A true advance in the knowledge of the organisms of fermentation was, however, first made, when the methods of physiological investigation were entered on. As before stated, endeavours were made very many years ago to employ methods of this nature; the entire absence of precautions in carrying out the experiments resulted, however, in complete failure, and a reaction then set in, which found

expression in the statement of Reess, in his work on the Ascospores of the Saccharomycetes (1870), in which he expressly emphasized the fact that he had taken no precautions to obtain pure cultures—to such a degree were these cultures held in discredit. In the course of the following year, however, the matter took another turn; and it is, perhaps, an almost unparalleled fact in the history of science, that a new method of investigation should have, in so short a time, not only cleared the road, but also arrived at such important results, both in pathological science and in our own special branch—results which have caused a revolution in many of the old-established dogmas.

The physiological investigation of micro-organisms has for its purpose the acquiring of an insight into the development and vital activity of their existence. The means employed for this is naturally to make such conditions for their growth and multiplication, that it is possible to observe the changes which are slowly taking place in the organisms, and the materials by which they are influenced. When we only desire to obtain a knowledge of the different forms under which any organism can appear during its development, the conditions are considerably easier than when we require a culture in bulk of individuals which are all derived directly from one cell of the species, in order that we may, by means of physiological, chemical, or purely technical researches with large numbers of the organism, arrive at an insight into the relation between the forms and the external influences of the organism, as well as into its entire vital activity. In the first case, we only require a culture in which the organism is able to grow undisturbed, quite neglecting the fact whether entirely foreign individuals or species are also present. In the latter case, however, an absolutely pure culture is necessary. Some examples will show this more clearly.

One of the naturalists who has furnished the most beautiful and complete contributions to the history of the mould-fungi is Brefeld. In order to assure himself that he had correctly explained the entire development of a mould-fungus,

he fixed directly under the microscope a spore of the plant, then caused it to germinate, and followed the growth of the germinating spore until this again produced spores. The cells were distributed through a drop of nutritive liquid, and, in order to prevent the liquid from drying up, a small quantity of gelatine was in some cases added ; this, at the same time, hindered the small bodies in the drop from shifting so easily from place to place as in a liquid substratum. One single cell was then brought under the microscope, and the work consisted in following with care and patience all the changes which took place in this cell. It is readily seen that it was quite indifferent whether cells of another species—bacteria, for instance—were present in other parts of the drop, so long as these did not interfere with the growth of the selected object. At intervals, drawings of the small plant in course of development were made, and in this manner the numerous beautiful drawings of the mould-fungi which we owe to this author were executed. This investigation was of a purely botanical nature. With the exception that the microscope had to be used as an aid on account of the smallness of the object, there is no difference between this investigation and the observation of the external phenomena of the growth of a flowering plant from the germination of the seed to the ripening of the fruit.

Cultures of the same kind may often give good results when we have the earlier-mentioned case before us, viz., a nutritive liquid in which secretions of various kinds have assumed a more or less deceptive similarity to different forms of bacteria, so that no certain knowledge can be acquired by an ordinary microscopic examination. A drop of the liquid is placed in the so-called moist chamber, in this instance that of Ranvier (Fig. 1).

This apparatus is constructed by grinding a very shallow cell in the centre of an ordinary glass slide ; this cell is surrounded by a deeper groove, which is intended to contain water ; the drop of the nutritive liquid, which must be very small, is placed in the middle of the shallow cell and covered with a thin cover-glass, which must be larger than the circular

groove; when the cover-glass is in place it is cemented fast by means of vaseline; the drop is thus spread out between

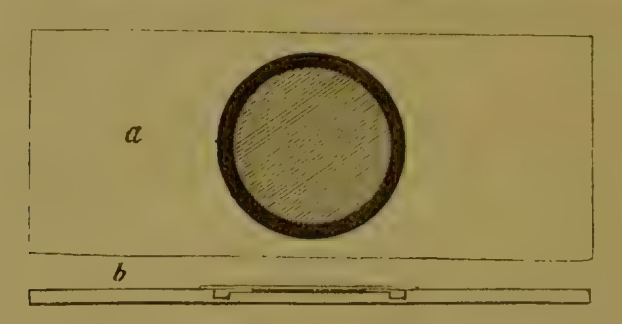


Fig. 1.—Ranvier's Moist Chamber.

a, seen in plan; *b*, in section.

the cover-glass and the shallow cell of the glass-slide, and protected from evaporation by the water in the circular groove.

Another form of moist chamber, the so-called Böttcher's chamber (Fig. 2), is prepared by tightly cementing a glass ring on an ordinary glass slide. In this space some drops of water are placed. A thin cover-glass, on the underside of which a small drop of the nutritive liquid containing the organism is placed, is fastened securely to the edge of the glass ring with vaseline.

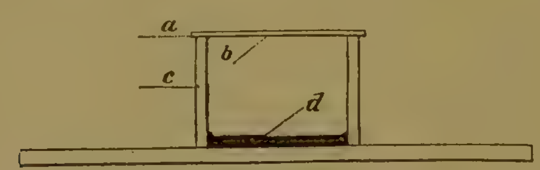


Fig. 2.—Böttcher's Moist Chamber.

a, thin cover-glass; *b*, layer of nutritive material; *c*, glass ring; *d*, layer of sterilized water.

Such an apparatus is brought under the microscope and the changes of the bodies observed from time to time, or it is placed in an incubator at a suitable constant temperature, and withdrawn at certain intervals and submitted to a thorough microscopical examination. The living bacteria

can then, if the nutritive liquid and temperature are favourable, be distinguished by their growth and increase from the organic and inorganic detritus.

Simultaneously with the above-mentioned cultures of Brefeld, a reform in the study of the micro-organisms of fermentation took place, and a review of the results of these new investigations constitutes the most important object of this work. It was required that cultures for large cultivations should be developed in such a way that physiological and chemical investigations on the development of the micro-organisms could be undertaken with a pure growth as a starting-point. Pasteur, Nägeli, Koch, and Hansen have developed methods in this direction, and we will now briefly consider their leading features.

Every fermentation, no matter whether beer, wine, spirits, vinegar, or other substances are produced, is a culture of living organisms, of "organized ferments," and the endeavours of the manufacturer are therefore directed—more or less knowingly—to obtaining, so far as the practical requirements will allow, a pure cultivation of the most favourable forms for the manufacture. Although in our time, with a better understanding of industrial requirements and aims, great advances in this direction have been made, yet certain fixed boundaries have always been established, which could not be overstepped on account of purely practical grounds; thus, the cultures on a practical scale will certainly never reach a point at which they are "absolutely pure cultures." It is one of the most salient features in the present development of the industries of fermentation, that, with a correct understanding of the importance of the organisms of fermentation as central points in the manufacture, we work for the purpose of emancipating in a continually higher degree the principal active, useful species from the action of the injurious forms. It is especially evident that this is of the greatest importance, since Hansen showed, with the systematically selected varieties of bottom yeast, that such a ferment grown in a pure state introduces a much greater certainty and uniformity in the process. We will, however, return to this point later on.

In researches in the laboratory, where the point in question is also the growth of cultures of organisms concerned in fermentation, much greater demands can be satisfied than in experiments on a practical scale. Here the requirements demand that we work with absolutely pure cultures, partly in small quantities, partly in such large masses that they may be interrupted at a given point in the course of the experiment in order to be transferred to the manufacturing process. Such conditions, which are wanting in the technical application, are sought to be realized in the laboratory, which is specially arranged for these investigations; and we will now briefly sketch these conditions, and the manner and form in which they are fulfilled. We will on purely historical grounds begin with the last members—the vessels and liquids, which receive the originally prepared small pure-cultures—and the expedients which are employed in the cultivation. It is necessary that, before the introduction of the inoculating substance, these flasks and liquids should be sterile, *i.e.*, free from all living, active germs, and also that the surrounding apparatus and the air of the place in which the work is performed should contain as few living germs as possible. The same remark applies naturally to the clothing and hands of the experimenter.

The requisite sterilization and disinfection, which, of course, should also be striven for technically, is naturally of quite a different nature to chemical purity. It can and must be carried out in a different manner. The pieces of apparatus, the nature of which will admit of the application of a temperature considerably above the boiling-point (for example, heating at 150° C. for two hours), can be directly sterilized in this way; metallic objects, as forceps, tongs, &c., and also glass apparatus can be quickly sterilized by ignition or by strong heating with a flame, and subsequent cooling in a germ-free space. Apparatus of inflammable material, when it has previously been mechanically purified, may be sterilized on the surface by being repeatedly and quickly passed through a flame. Nutritive liquids are brought to a state of sterility by boiling in the ordinary way for a length of time which

depends on the nature of the liquid and on the materials which were used for its preparation. It is necessary to take precautions during the cooling that only germ-free air can come in contact with the liquid. We will return to this later. It is known that the vegetative stages of growth of bacteria cannot withstand a heat of 100° C., whereas the spores of many species are considerably more resistant. It is often necessary to boil for many hours before they are killed. It is very noteworthy that when the spores of the hay bacillus are submitted to a short boiling, they appear by this treatment to be stimulated rather to a greater activity than to the reverse.

Certain liquids cannot be heated to the boiling point, since at that temperature such changes in their composition take place that they are finally rendered useless. For example, this is the case with blood-serum, which is employed in certain bacteriological studies, and used in a gelatinous condition. This material when heated to 100° C. becomes fluid and does not again solidify, and it is therefore necessary to proceed in a different way in order to obtain it sterilized in the solid state. It was observed that a temperature of 58° to 62° C. was sufficient to kill the vegetative bacteria which developed in blood-serum. By this treatment only the spores of the bacteria remain unkilled. If the gelatinized serum is then placed for a day or two in a thermostat, at a favourable temperature for the development of the spores, *i.e.*, about 30° C., the greater number of these germinate, and the new vegetative rods can then be killed by again raising the temperature to about 60° C. This process is repeated several times, and the solid mass will then remain sterile for an unlimited time, provided no germs from the air obtain admittance. This procedure, which is also used for the sterilization of milk, and which was discovered by Tyndall, has been thoroughly confirmed by R. Koch.

Another mode of killing the disturbing organisms is by the employment of disinfecting substances, which act as poison on the organisms. These substances also find considerable employment for practical purposes. The limits for the use of

these poisonous substances must be determined in each individual case. Since it is probable that the manipulation of such poisons may act injuriously on the workmen, the question has been determined in how great a state of dilution they can be used in order to act to the fullest extent. In communications to the Royal Sanitary Board in 1881, R. Koch has given the results of a considerable number of investigations of this nature which were made from hygienic considerations. The various disinfecting substances were tested for their power of killing spores of bacteria, spores of mould, yeast-cells, and bacteria. For practical reasons, however, Koch at the same time endeavoured to ascertain what quantity of the substance under examination sufficed to stop the development of micro-organisms in a favourable nutritive liquid. I give here in brief, from the published researches of Koch, the results obtained. Carbolic acid did not prove to be such a superior disinfecting substance as it is generally considered to be. A 5 per cent. solution destroyed the power of development of the spores of the bacillus of anthrax only after forty-eight hours, whilst the *Bacillus anthracis* itself was killed in two minutes by the action of a 1 per cent. solution. A solution of 1 in 850 sufficed to stop the growth of the latter; wetting the spores of *Bacillus anthracis* five to seven times with a 5 per cent. solution caused a retardation of their development. In oily or alcoholic 5 per cent. solution, carbolic acid was absolutely without action on the spores of this bacillus. In the form of vapour, carbolic acid acts more strongly, yet the action of carbolic acid vapour at 75° C. for two hours did not destroy the power of development of the above-mentioned spores. Sulphurous acid is not able, except under very favourable conditions, to kill all germs; the same holds good for calcium bisulphite and zinc chloride. Chlorine, bromine, and corrosive sublimate (mercuric chloride) are, however, disinfectants certain in their action. The sublimate, according to Koch, acts fatally upon all germs in a proportion of 1 in 1,000, in most cases even in 1 in 5,000. This statement must, however, according to the new investigations of Johan Olsen, be considerably modified; Olsen found that with some

of the mould-fungi, namely, *Penicillium glaucum*, *Aspergillus niger* and *clavatus*, dilute solutions of corrosive sublimate showed, without doubt, a retarding action on their growth and development inversely proportional to the strength of the solution; but that it required a solution of 1 in 2,000 to completely stop the growth of *Aspergillus niger*, 1 in 1,000 for *Aspergillus clavatus*, and a concentration of 1 in 400 for *Penicillium glaucum*. Many Schizomycetes also (the organisms of puerperal fever, abscesses, and putrefaction) germinated and grew, although more slowly than usual, on slices of potatoes, even when these were saturated with a solution of sublimate of 1 in 500, whilst a solution of 1 in 300 is required to absolutely stop their growth; indeed, after the action of a pure solution of sublimate of 1 in 1,000 for half an hour they retain their activity, and it is only after treatment with a solution of 1 in 500 that this is stopped. The duration of the action is of great importance with the Schizomycetes. A solution of corrosive sublimate of 1 in 1,000 can only arrest the development under certain conditions, namely, by the continuance of the action for at least one hour, and by the withdrawal of suitable nutriment; whilst this solution, with a shorter period of action and in the presence of nutritive material for the organism, is not to be relied on. In the physiological laboratory this substance finds employment on all sides, but much less in technology, on account of its strong poisonous properties. We must, therefore, recommend chlorine and its compound with lime as an excellent, easily used, and cheap disinfecting substance. In those physiological laboratories in which it is especially necessary to guard against the invasion of foreign germs, an alcoholic solution of salicylic acid also finds useful employment (it is always used by Hansen in the Carlsberg Laboratory for the purification of the working benches). The action of this substance in dilute solution in checking fermentation is generally known. According to the researches of Gayon and Dupetit, basic bismuth nitrate exercises a great retarding influence on the bacteria which set up bye-fermentations in an alcoholic fermentation, whilst the Saccharomycetes are not affected by it.

We will now return again to our subject, and give some instructions regarding the preparation of the vessels in which the cultures are made in the laboratory, and regarding the choice of the nutritive substrata.

The chief condition required from the vessels in which the cultures are made is that they shall be closed to every contamination from the outside. Pasteur's flasks satisfy this demand in the highest degree. The illustration (Fig. 3)

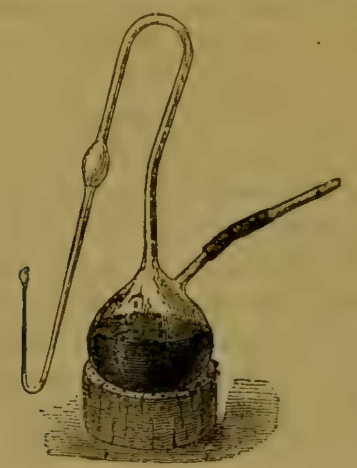


Fig. 3.—Pasteur's Flask.

shows this flask in the slightly modified form, as now used in the Carlsberg Physiological Laboratory directed by Hansen. When the nutritive liquid is boiled, the steam first finds its way out through the wide straight tube, which ends with a piece of india-rubber tubing ; when this is closed the only outlet for the steam is through the drawn-out neck. After some time the flask is taken from the sand-bath, and the end of the drawn-out neck closed with a small plug of asbestos. The sterilization is then complete, and the contents of the flask should remain for years without any change taking place. During the cooling, and the consequent indraw, the air is filtered through the asbestos plug, and even in the event of this filtration not being complete, the germs which are carried through are deposited in the lowest part of the bend, or, at the most, do not pass the enlargement of the thin tube, and therefore do not come into contact with the

liquid. When the liquid in the flask is violently shaken, or when it is emptied away by the straight tube, and it is wished to still keep the flask free from infection, the lower part of the narrow tube must be heated in a flame. If the flask is opened and placed in connection with another flask, it is necessary to do this either in a small, germ-free space or the tube and stopper must be made hot with a flame before and after the operation; when this is done, and the operation is quickly performed, there is seldom any danger of contamination.

During the last few years various other flasks and vessels have been brought into use, notably the Chamberland flask (Fig. 4), the neck of which is closed with a ground cap which



FIG. 4.—CHAMBERLAND FLASK.

terminates above in a short open tube; this tube is filled with tightly packed, sterilized cotton-wool. Salomonsen uses flasks with a short conical neck, to which an india-rubber tube with several small plugs of cotton-wool is attached. Pasteur's flasks are most unquestionably indispensable in certain directions; for instance, in physiological investigations, especially on alcoholic fermentation.

With regard to the nutritive substrata, the problem naturally consists in always finding those which are most suitable for the respective organisms. If these also possess the advantage of being *per se* less favourable for the development of concurrent forms, it is a great point gained. It is, of course, also

the rule, when comparative investigations are made in various directions, that the nutritive liquid must always remain the same. For the investigation of alcoholic ferments Hansen principally used hopped wort from the filter-bags; in special cases in investigations of this kind, yeast-water, with the addition of glucose or a solution of saccharose or other carbohydrates in water was employed. If it is desired to use a solid nutritive material, the liquid is mixed with 5 to 10 per cent. of gelatine. Similar liquids, or, more frequently, meat-extract with an addition of peptone, are employed for bacteriological investigations; the latter mixture is neutralized with sodium carbonate. Either gelatine or Agar-Agar is used for the purpose of rendering the medium solid. Solid nutritive substances are the best for the study of mould-fungi, in most cases preferably sterilized black bread. If liquids are employed, the most suitable for the purpose are beer-wort, fruit decoctions, or mixtures of sugar with an addition of tartaric acid or tartrates. Pasteur used liquids exclusively as substrata in his celebrated investigations on the organisms of fermentation ("Études sur le Vin," 1866 and 1873; "Études sur le Vinaigre," 1868; "Études sur la Bière," 1876). Later the solid substrata were very extensively employed, and in this respect Koch has given many practical illustrations.

We have now shortly explained how our micro-organisms are cultivated, how guarded against contamination from the liquid itself, from the vessels and apparatus, and to a certain extent from the air and the experimenter. We have now before us the first and most important question: How do we obtain the first absolutely pure cultivation which is introduced into our flasks? As mentioned above, I began on purely historical grounds to sketch the conditions for the preservation of the pure cultivation, because these were known long before the preparation, with certainty, of the pure culture itself had been reached.

In this respect it will be instructive to see how we have advanced step by step, and we will again for the moment take up the subject historically, and see where really rational endeavours have been made for the attainment of this object.

In his "Études sur la Bière," published in 1876, Pasteur devoted two leading chapters to the culture of micro-organisms in a pure state; in the fourth chapter he mentions the forms of mould-fungi, and the organisms which form a film of mould on liquids containing sugar; and in the fifth chapter he treats of the yeast-fungi. We will take some examples from these chapters. In order to obtain a pure cultivation of the most common mould-fungus—*Penicillium glaucum*—Pasteur adopted the following procedure:—The mould was allowed to develop on a suitable material (an operation which presents no difficulty, since this form is widely diffused in nature), and when it had raised its fruit-bearing hyphæ from the substratum, and had developed a considerable quantity of conidia, which could be distinguished by the naked eye as a fine powder, a small piece of platinum wire, which had immediately before been passed through a flame, was drawn over the growth in such a way that it touched this fine powder, and then quickly dropped into one of the flasks already described. In this a pure growth of the mould should then be developed. The author, however, himself raises an objection to this method, namely, that a great danger to the purity lies in the fact that the air may have carried foreign cells to the first cultivation, and these would be removed and sown at the same time as the mould spores. He therefore goes further, and gives another more reliable method. A number of the ordinary flasks with a single straight neck were taken, and the necks drawn out to a fine point (Fig. 5); they were then half filled with a suitable nutritive liquid and sterilized. Whilst they were boiling the upper point of the neck was fused, and, consequently, no air could obtain admittance to the flask on cooling. A number of flasks prepared in this way were then taken to a suitable place, the extreme point of the neck broken off with a pair of pincers previously purified by passing through a flame, and the air with its living germs allowed to rush violently into the flask, after which the point was again fused. "It then often occurs that *Penicillium* alone appears, so numerous are the floating conidia of this mould-fungus in

the air. We have clearly enough under these conditions a field of conidia without the slightest foreign admixture.'

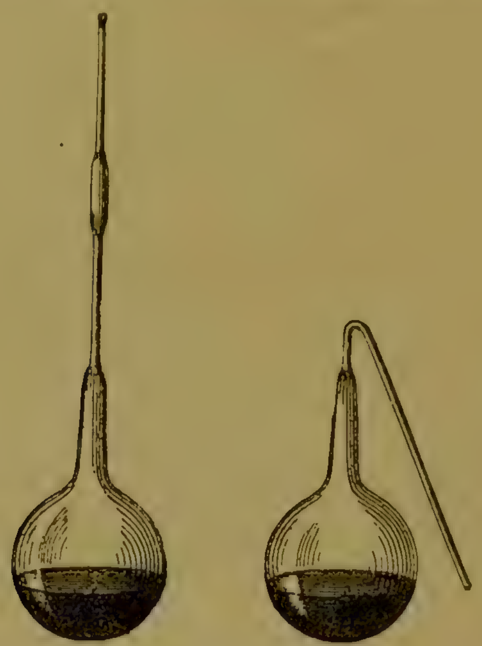


FIG. 5.—VACUUM FLASKS.

From one of such flasks some conidia were then transferred to one of the other flasks, and "in this way we have the most certain means of cultivating the conidia of *Penicillium* free from impurities." Closely examined, the method in the latter case does not appear markedly better than in the former; for, as the air in the former case can convey every possible contamination to the growth of mould which is touched with the platinum wire, so in the latter case the same holds good, only to a smaller extent. Such a cultivation cannot be used for a bulk culture, since the purity is not secured from the commencement.

Pasteur mentions, in the fifth chapter, a similar method for the preparation in a state of purity of the alcoholic ferments: a small quantity of yeast was dried and triturated with powdered gypsum. This fine powder was thrown into the air from the highest possible position, and whilst the particles were falling, a series of flasks was opened as in the

former case, and it might then happen by chance that yeast-cells, which were finely distributed in the cloud of dust thus formed, would be drawn singly into some of the flasks. It was then, as with the mould-fungus, a chance which produced a pure culture. And since at the time when Pasteur's work appeared there were no means of determining the purity or otherwise of the growth, the above-mentioned method was always very uncertain.

The two examples above mentioned possess a very great interest, since we see in the experiments of the celebrated French naturalist perhaps the first steps towards the so-called "fractional cultivation." The theory, which led to these researches, was that by means of some material—in the above-mentioned cases the air—so fine a distribution of the microscopic germs was brought about, that it was possible to cause them to be sown singly. These methods have been further developed during the last ten years, and we will now examine these developments in their different stages.

Before, however, we proceed to do this, I must briefly mention two other methods, which aim at the same end, and still find frequent employment. These are based, on the one hand, on the possibility that different species of organisms are able to offer different resistances to the same inimical attack on their vital activity, and, on the other hand, on the fact that different species are able to behave somewhat differently when the same nutritive material is offered to them. These methods were particularly used by Pasteur, in order to prepare pure cultivations. A method generally employed for the preparation of a pure culture of the very widely diffused hay bacillus consists in allowing a hay-infusion to stand for some days, then boiling it for a short time, and again placing it on one side for the further development of the spores. Since the spores of *Bacillus subtilis* withstand a temperature of 100° C., whilst other vegetative bacteria are killed under these conditions, the surviving spores of hay bacillus are able to develop and give a pure culture—so long as no other spores equally capable of resisting this temperature are present in the infusion.

The second consideration, which is also frequently relied on as a means for the preparation of pure cultures, is the different behaviour of species to the same nutritive material (Pasteur, Klebs). If, in a mixture of micro-organisms, there occurs one species which has a special liking for the nutritive material in which the mixture is sown, then, when a trace is continually transferred from the successively developed growths to new flasks with the same nutritive material, the species in question always develops at the expense of the others, and a time will arrive when this species appears to have conquered the whole territory. We must, however, here again consider the possibility that the other organisms are possibly only checked or strongly retarded in their development; the favoured species uses up the food more and more, and sooner or later enters into a condition of weakness, perhaps checked and poisoned by the substances which it has itself produced during the period of active absorption of the nutritive material—and then where is the certainty that the other species do not now find more favourable chances for their development? An instance of this kind is known with certainty in the practical application of fermentation. The case may also occur when two or more species show an equal power of multiplication under the above-mentioned conditions.

After this digression, which is not only a link in the history of the advances, but also of the mistakes of this branch of science, we will return to the so-called fractional cultivations—methods of dilution. In this respect Nägeli has contributed some interesting communications regarding the bacteria. In order to bring about the sowing of an individual cell, it is first necessary to know how many individuals there are in a determinate volume of the nutritive liquid. He counted these, and from the number found calculated that the liquid, when diluted to a certain extent, contained one cell in a given small volume.

Infection with this volume was, therefore, the foundation of a true pure cultivation.* The goal is here approached

* For example, in a small drop of putrefactive liquid, which measured

somewhat nearer, and, provided that the counting is correctly made and the experiment carefully carried out, the desired result can be arrived at by this method. It is self-evident that the conditions must always admit of some doubt, for an accurate estimate of the number of individuals in a bacterial growth is impossible with the aid of the methods at present known. Even when this is possible, a new question presents itself. How are the flasks which have received one cell to be distinguished from those flasks which, in spite of the calculation, were infected with several cells? No method for this purpose has been found for bacteria; only for yeast-cells has such a method been proposed by Hansen, namely, by means of the yeast-specks.

Another method was introduced by the celebrated R. Koch. He employed as a diluent liquid first sterilized water, and afterwards a nutritive solution to which so much gelatine was added that it was solid at the ordinary temperature. By this means the fixture of the germs was attained, and they were able to develop free the one from the other. The procedure consists in taking a trace of an impure culture and transferring it to a large quantity of sterilized water. From this a small quantity is transferred to a flask which contains, for instance, a mixture of meat-broth and gelatine warmed to 30° C. The flask is shaken in order to distribute the germs, and the contents poured out on a large glass plate, which is at once covered with a bell-glass. The gelatine then quickly sets, and the germs remain enclosed in the solid mass. They grow in a few days to colonies—points or specks, which are visible to the naked eye. There is here a great probability that the germs have been enclosed singly in the gelatine, and if such a small colony is transferred to a sterilized flask, a pure cultivation is, at least in most cases, obtained with the bacteria. The purity of the specks

0·03 c.c., 500,000 individuals were counted; the drop was added to 30 c.c. of sterilized water, and was thus diluted 1,000 fold; the liquid was thoroughly shaken, and a drop again withdrawn and diluted with 30 c.c.; the original liquid was thus diluted 1,000,000 times, and every second drop contained one organism.

in the gelatine is ascertained, according to Koch, partly by their appearance, colour, form, &c. An absolute certainty is, naturally, not attained by Koch's method.

If now we are given the task of preparing pure cultures of the yeast-fungi, and especially of the Saccharomycetes, none of the above-mentioned methods can be freely recommended. The different varieties, when transferred to Koch's gelatine plates, produce colonies which do not show any, or, at the best, only very slight differences. Direct experiments made by Hansen have shown that in spite of all care a speck is here and there found in such gelatine cultivations, which is formed from different varieties, two or more cells having remained enclosed together. How can we remove this difficulty? Hansen has supplied the answer: By taking care that the layer of gelatine, formed by the solidified nutritive liquid, is prepared in such a way that the position of the isolated cells can be observed under the microscope; further, that the position of these cells can be accurately marked, and also that the individuals can be seen to develop step by step to a colony. The glass plate, which in Koch's experiments is of considerable size, consists in this case of a round cover-glass of about 30 mm. diameter. This is fastened to a glass ring, which again is cemented to a thicker glass, and forms the previously described moist chamber (Fig. 2, p. 8), which is adapted to the purpose, and carries a layer of solid gelatine on its inner upper surface. The great point in the method now to be described is, that at no stage is anything done at random. The cells must be so greatly divided that they occur with only comparative rarity in the gelatine layer; the chamber is then either allowed to remain under the microscope, in order that the multiplication of the cells may be directly followed, or the positions of the well-isolated cells are marked, either by dividing the glass cover in small squares, or in any other way, and the apparatus is placed in the incubator until the colonies are completely grown. When this happens the colonies are transferred with a small piece of platinum wire, which has previously been ignited, to the Pasteur flasks. The cultures are during this transference an instant in the air, and are here

exposed to contamination. The danger of contamination at this single weak point is reduced to a disappearing minimum, to infinity, if the above-mentioned operation takes place in a small, enclosed, germ-free space; for instance, in a small cupboard with glass walls, which is sufficiently large to admit the apparatus and hands of the experimenter. The cupboard must be provided with a small door, through which the hands may be introduced; the walls are moist on the inside, the hands of the experimenter freed from all living germs. In this way the transference of the colonies is performed with all possible security. In most cases, however, the entire process is carried out in an ordinary clean room. From the first flask the culture can be transferred without a possibility of contamination to a continually increasing number of larger flasks, and, according to universal criticisms, we can then assert that Hansen's method approaches the desired end as nearly as is possible. We will consider later the second, and quite as important, part of the operation—the control of our pure culture.

In the pressed yeast and spirit manufactures it is of importance to determine the multiplying capacity of the yeast-cells during the growth of the yeast. This must naturally be effected by a direct counting of the number of cells which occur in a determinate volume of the fermenting liquid at different stages of the fermentation. Researches of this nature have been undertaken by Delbrück, Durst, Hansen, Hayduck, and Pedersen, whilst Fitz has applied the same method to bacteria.



The counting is performed by means of an apparatus constructed by Hayem and Nachet (Fig. 6), which was first employed for counting blood corpuscles (hence known as hæmatimeter). The late Professor Panum, of Copenhagen, was the first to employ this apparatus for counting the number of micro-organisms, in order to determine their multiplying capacity. The hæmatimeter consists, as the figure shows, of a glass slide, on which a cover-glass of

known thickness (0·2 mm., for instance) is cemented, and which has a circle cut out of its centre.

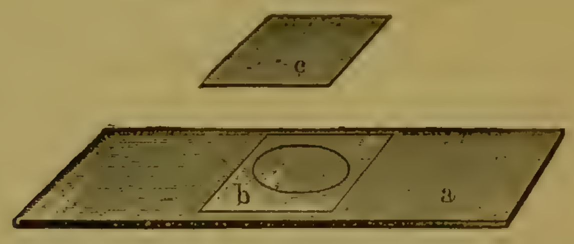


FIG. 6.—HÆMATIMETER.

a, glass slide; *b*, cemented cover-glass with circular opening; *c*, cover-glass.

A small drop of the liquid containing the cells is brought into this hollow, a cover-glass is placed over the opening, and rests on the cemented, perforated cover-glass. The drop of the liquid must not be so large that this pressure causes it to flow out from the cut-out space enclosed: it must, however, be high enough to be in contact with the superincumbent cover-glass. The thickness of the layer of the liquid is then known. In order to determine the other two dimensions, and thus be able to work with a given volume of liquid, one of the generally known micrometers, consisting of a thin piece of glass on which a number of small squares are engraved, is brought into the eye-piece of the microscope. The actual value of each of these squares is known when a given system of lenses is employed, and, therefore, when the square is projected on the object, a small prism of known volume is defined. In certain cases it is more useful to have a fine system of small squares of known diameter engraved on the glass slide itself at the bottom of the cavity (Zeiss, of Jena, has made a chamber of this sort from the instructions of Professor Thoma); this also improves the microscopic definition of the cells which are on the bottom of the chamber.

When only a determination of the multiplying capacity of the cells is desired, or repeated observations of the number of cells in the same volume of liquid, it is quite superfluous

to determine the size of this volume; it is then only necessary to always work with the same volume.

It is always necessary that the sample taken be a fair average one. In most cases this must be diluted and thoroughly agitated for a long time, in order to obtain an equal distribution of the cells; the specific gravity of the liquid must also be such that it will allow the cells to remain suspended in it for a short time. A small drop is then withdrawn with a capillary tube, transferred to the counting apparatus, and covered with a cover-glass. The apparatus is now allowed to remain at rest for some time; the cells are then able to settle at the bottom of the defined space, and on this account the specific gravity of the liquid must not be greater than will allow this to take place in a convenient time. Both these requirements are generally satisfied by the wort used for industrial operations.

If it is found that the determinate volume still contains too many cells to be counted with certainty, the liquid must be further diluted. This may also be advisable for other reasons: partly to prevent the formation of froth, which generally forms abundantly from the violent agitation, and partly to isolate the single cells which, in wort, are frequently collected together in colonies or large masses, and are not always separated by shaking, and, finally, in order to bring about the discontinuation of the fermentation and the multiplication of the yeast-cells after the beginning of the experiment.

Hansen found that dilute sulphuric acid (1 : 10) completely answered these requirements; hydrochloric acid, ammonia, and caustic soda may also be used, but they are not so good. If a very great dilution is required, distilled water can be added, after the addition of 1 to 2 volumes of dilute sulphuric acid.

When the different volumes of liquid are mixed with accuracy, and care taken that the cells are thoroughly distributed by violent and prolonged shaking, the determination can be made with great exactness. Two similar dilutions must always be made, and samples from each taken for

counting. Experiments must also be made in order to determine of how many squares the cell contents must be counted in order to arrive at a true average. Such a counting and determination of the average number is continued until the increased number of squares has no influence on the average value. The number of countings necessary, and the accuracy generally, depend on the experience of the observer and the care exercised. Hansen found that under ordinary circumstances it was sufficient to count the cells in 48 or 64 small squares.

CHAPTER II.

THE EXAMINATION OF AIR.

As the water was formerly considered to be one of the enigmatical factors in the fermentation industries, and had frequently to bear the blame of irregularities which could not be explained in any other way, so many peculiarities of a certain nature in the results obtained have been always considered to originate from the air. In this idea was involved an ill-defined misgiving that this invisible air contained substances which act prejudicially to our efficiency; of what nature these substances were, and especially how it was possible to obtain a closer knowledge of them, was, until the most recent times, entirely wrapped in obscurity. Chemical investigations of the air were made more than a century ago. In the year 1774, Priestley and Scheele discovered that the atmosphere consisted of oxygen and nitrogen. In 1804, Gay Lussac and Humboldt showed that the proportion between these two substances was the same everywhere; later it was found that the air also contained water-vapour, carbonic acid, ammonia, and nitric acid, and in individual localities varying quantities of chlorine, sulphuretted hydrogen, marsh gas, &c.

Gradually another new factor was added; it was unmistakably proved that the air is not everywhere equally favourable to the human subject; there might possibly be found something which attacked our organism; this unknown quantity was called "Miasma" (mixtures); the word was taken in a purely chemical sense. Since, however, these miasmata were not traced further, science was thereby not advanced one step.

From entirely independent workers, a new view of the

contents of the air was evolved. This related to the presence of micro-organisms, and within a short time it was clearly established that these occur everywhere in the air; it was also rendered probable that they play an important part in the constitution of the air. That these micro-organisms are also of considerable importance for the fermentation industries was especially shown by Pasteur when he proved that the atmosphere contained not only bacteria, but also alcoholic ferments.

The questions which then arose were—What is the nature of these germs floating in the air? In what degree and to what extent do they occur in space? Do their number and nature vary with the different seasons of the year? And finally—Are they actually able to interfere in an energetic manner in technical operations?

It will be of interest to glance at the different methods by which it has been sought to analyse the air with regard to its germs.

The majority of the analyses of air have been made for the purpose of throwing some light on the mysterious obscurity which envelops most contagious diseases, nearly all of which are known to be conveyed by the activity of microscopic organisms. For the organisms of fermentation, the investigations of Pasteur, and later those of Hansen, come to the front. The French *savant* states that these germs are always floating about in the air; as a rule, however, they are found in much larger quantities in the dust which settles on the vessels employed. The true alcoholic ferments are present in comparatively small numbers in the air, whilst the germs of the mould-fungi are more frequent: he further shows, as also did Tyndall later, that the germ-contents of the air varies, both with regard to the number as well as the nature of the germs. Pasteur arrived at these results by exposing in open shallow dishes different varieties of beer-wort, wine-must, or yeast-water containing sugar; after some time their contents were examined for microscopic organisms. Later, Pasteur employed for this purpose the vacuum flasks previously described (Fig. 5, p. 18).

Without doubt, the investigator who in the last few years has undertaken the largest number of air analyses is Miquel, the director of the laboratory specially set apart for this purpose at Montsouris, near Paris.

He performed his first experiment with a so-called aëroscope (Fig. 7), which is constructed in the following manner:—From the top of a bell-glass, A, proceeds a tube, C, by

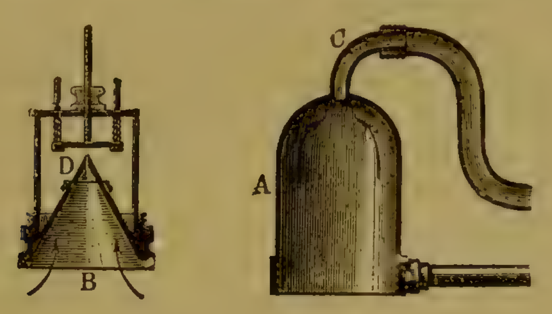


FIG. 7.—AËROSCOPE.

which air is aspirated, thus causing it to pass through the bell-glass. To the open end of the latter is screwed a hollow cone, the mouth, B, of which points downward; in the apex of this cone, D, there is a very fine opening through which the aspirated air is drawn, and immediately over this opening is fixed a thin glass plate covered with a mixture of glycerine and glucose. The viscous mixtures remove what is carried in by the air, or, at least, to a very great extent. The micro-organisms intercepted here are distributed as equally as possible on the glass plate, and counted under the microscope. This method is so far defective that no information is obtained on the most important point, namely, which, and how many, of the intercepted germs are actually capable of development. In order to learn this, Miquel employs another method. He collects the air-germs in sterilized water. A large number of small flasks containing nutritive liquid (for instance, meat-extract made either neutral or slightly alkaline) are then infected with a certain small quantity of the well-agitated diluted liquid. The aim here is, to ascertain by experiment how far the dilution of the liquid infected by the air must be carried in order that a large

number (one-half, for instance) of the small flasks which have been infected by this liquid shall remain sterile; there is then, therefore, a certain probability that in each of the remaining flasks, in which growths have developed, only one germ has been sown. A simple calculation will then, of course, show how many germs, capable of development in the medium employed, there were in the measured volume of air aspirated through the original flask.

By these methods of investigation, Miquel found that similar volumes of air from the same locality contained at different times a different number of bacteria. A prolonged rain purifies in a high degree the air from bacteria, and their number continually diminishes whilst the earth is moist, after which they again gradually increase with the drying of the ground. In the dry seasons of the year the number of bacteria is then, as a rule, the greatest, whilst the mould-fungi, which thrive best in moisture, and whose organs of multiplication project upwards, are the most plentiful in the air during the periods of moisture. The purest air is found in the winter time; the air in towns is less pure than that outside the towns; germ-free or nearly germ-free air is found at sea and on high mountains. In certain localities—hospitals, for instance—the air is very rich in bacteria; in one case even fifty times richer than the air in the garden at Montsouris.

In order to introduce air-germs into liquids, Miflet has used in Cohn's laboratory a water-pump (aspirator), which draws the air through several flasks containing nutritive liquids; these flasks are then placed in an incubator for development, and afterwards microscopically examined. The aspirator consists of a large, air-tight vessel, filled with water, and connected at the top with the series of flasks; near the bottom it is provided with an outlet tap. When the water is allowed to flow out through the tap, air is drawn in at the top, and must consequently first pass through the nutritive liquid in the flasks. It was shown generally by these investigations that air contained numerous active bacteria. The most important defect of this apparatus, which leads air

through a liquid, is that in this way only a portion of the air-germs are removed. As the air rises through the liquid a portion of the germs travel with it, and are thus carried off. This observation, made by Miquel, is of practical importance, since it shows that the filtration of air through layers of liquids—sulphuric acid, for instance—is not sufficient under all conditions. These depend both on the rapidity of the current of air, and on the thickness and nature of the layer of liquid.

An entirely different method for the examination of the organisms of the air is that employed in Koch's laboratory, and more completely developed by Hesse. A glass tube of about 1 metre (39·37 inches) long and 4 to 5 cm. (1·5 to 2 inches) wide is closed at one end with a perforated india-rubber membrane, over which another non-perforated cap is bound; a little fluid gelatine mixture is then poured into the tube, after which the other end of the tube is closed with an india-rubber bung, through which is inserted a glass-tube closed with cotton-wool. The whole apparatus is then heated sufficiently to render it sterile, after which the tube is placed in a horizontal position, so that the gelatine sets in a layer in the bottom of the tube. When the air is to be examined, the outer india-rubber cap is removed, and air slowly drawn through the tube by means of an aspirator attached to the other end of the tube. The germs in the air then settle down on the gelatine, and after the aspiration is concluded the tube is again closed and placed in the thermostat, where the germs produce visible colonies which are easily counted. The results show that with a sufficiently slow current of air the bacteria develop in the more forward part of the tube, whilst the mould spores develop much further along the tube. In spite of the fact that this method has its advantages, it is still in some respects inferior to the previously mentioned method; as a rule, it yields, to a still greater extent, numbers which are too low, for it appears that the solid nutritive gelatine is often not so favourable as a liquid medium for the development of many of the floating air-germs, which are often very desiccated; many of the germs are therefore not able to form colonies, and are consequently not counted.

Percy Frankland and Carnelley have each devised a method for the examination of the organisms of air based upon Hesse's method. The former aspirates a known volume of air through a small glass tube containing two sterile plugs consisting either of glass-wool alone, or glass-wool coated with sugar. The two plugs are then transferred to flasks containing melted sterile peptone-gelatine, and well disintegrated and mixed with the gelatine, after which the latter is allowed to set: on incubation at 20° C., the colonies appear and may be counted. Carnelley employs a conical flask with a thin layer of solidified nutritive gelatine on the bottom; air is slowly aspirated through the flask, and the organisms in the air settle on the gelatine, develop into colonies, and may be counted.

Hansen's investigations of the air were made in the period between 1878 and 1882. The most important object of these investigations was to obtain information for the fermentation industries. As is known, his beautiful researches on *Saccharomyces apiculatus* (1880) were partly based on work of this nature. Since the question concerned the organisms which occurred in brewing operations, the choice of a nutritive liquid was easily made, namely, a fermentable saccharine liquid of the same nature as that employed in large industrial operations, and as the researches were carried out in the laboratory attached to the brewery of Old Carlsberg, nothing was more natural than the use of the wort for bottom-fermentation beer as prepared in this brewery. The apparatus employed was either ordinary boiling flasks closed with many layers of sterilized filter paper, the contents of which were boiled for a certain time, or flasks of the same kind as Pasteur's vacuum flasks (Fig. 5, p. 18), the necks of which were drawn out to a fine point, and which were closed with wax whilst boiling. A little below the point a notch was made with a file, in order that the point might be easily broken off when it was desired to admit the air.

When these flasks had become well filled with the air of the locality it was wished to examine, they were again closed with wax and thoroughly shaken in order to mix the contents of the infiltrated air with the liquid. They were then put

aside for a shorter or longer time—up to six weeks—and examined microscopically.

In these investigations Hansen often found that the wort was preserved bright and apparently unchanged, even although a growth had taken place. Therefore it is not possible to rely on the examination with the naked eye alone. He names the following as forms which, when they are present in a feeble state of growth, cannot be detected macroscopically :—*Aspergillus*, *Mucor*, *Penicillium*, *Cladosporium*, *Mycoderma aceti* and *Pasteurianum*, and, finally, *Saccharomyces Mycoderma*. Even when these micro-organisms have formed vigorous growths, the above-mentioned nutritive liquid has remained bright.

It was further shown that Hansen, by the use of these flasks, often obtained pure cultures, since only one species was drawn into the flask with the air. It happened very seldom that three or four species penetrated into the same flask. This arises from the fact that only a very small volume of air enters each flask. The advantages of this are evident, because a true knowledge of these germs can only be obtained when they have developed. In cases where several germs penetrate into the same flask, the strongest germ by its growth will, in all probability, prevent the development of the others, so that these will not be detected by a later examination. At the same time, this advantage necessitates the opening of a large number of flasks, which makes this method of investigation cumbersome and costly. Since these flasks only tell what was present in the air at the moment of opening, ordinary boiling flasks were also used to give supplementary information. They were allowed to stand exposed to the air of the same locality for a longer time, in some cases as long as forty-eight hours. As a simple method, Hansen usually collects the organisms by aspirating the air through water, and then adds small quantities of the water direct, or after suitable dilution, to a series of flasks containing wort.

After these preliminary remarks we will give a short *résumé* of Hansen's results.

He confirms the statement, first made by Pasteur, that the

air may, at neighbouring points at the same time, contain different numbers and different varieties of organisms; Hansen also confirms the correctness of this rule for places lying close together in the same garden. He states, as other characteristics significant of the distribution of organisms, that those forms, for instance, which in the first half of July commonly occurred under the cherry trees in the garden, were quite absent in the latter half of the same month in the same place; and that those organisms which at one time were to be found under the cherry trees, but not under the grape vines, were a little later only to be found under the latter. As a proof of the inequality of distribution of the organisms, it is shown that the flasks opened in the same series of experiments and in the same place often had the most diverse contents.

The experiments with the vacuum flasks have further taught us that the organisms often make their appearance in groups or clouds, with shifting intermediate spaces, which are either germ-free or only contain quite isolated germs. Since the organisms cannot increase in the air, but have their place of growth in the earth, it follows that their presence in the air must be dependent on the condition of the surface of the ground, which again depends, in certain respects, on the weather.

Hansen's numerous analyses have further demonstrated the fact that the Saccharomycetes occur comparatively seldom in the dust of the air. Their number in the air increases from June to August in such a way that the flasks were frequently infected with these ferments at the end of August and beginning of September, after which a decrease took place. Those micro-organisms of this nature which enter the flasks at other times of the year in the open air must be regarded as unimportant and accidental, and therefore falling outside the true principal rule. Since most varieties of Saccharomycetes have in all probability their winter quarters in the earth, like *Saccharomyces apiculatus*, and their places of growth on the sweet, succulent fruits, therefore these latter must, as it appears from the above, be considered as the most important source of the contamination. At the

same time of the year bacteria are found in the largest numbers. This, therefore, constitutes an important danger in technical operations, since the wort, which is spread in thin layers on the open coolers, is exposed at the above-stated season of the year to a great contamination from the germs of the air.

Bacteria are found in somewhat greater number in the flasks than the Saccharomycetes, and the mould-fungi occur in still greater quantity. Amongst these Hansen mentions *Cladosporium* and *Dematium* as especially prevalent, and after these *Penicillium;* less frequent were *Botrytis*, *Mucor*, and *Oidium.*

After Hansen has thus stated which of the micro-organisms found in the open air are able to develop in flasks with sterilized wort, he proceeds to communicate his results of the examination of different localities in the brewery.

When grains are allowed to stand in the open air, they evolve, as is known, acid vapours, and since they always contain a rich growth of bacteria when they remain exposed for a short time, the following question at once suggests itself: What is the condition of the air found in the neighbourhood of the heaps of grains? It was shown that, of the flasks opened in these vapours, only 30 per cent. were contaminated, of which 3·6 per cent. consisted of Saccharomycetes and 2·4 per cent. of bacteria, whilst parallel experiments in the garden gave a contamination of about 44 per cent., of which 8·5 per cent. were bacteria. The air near the grains was therefore poorer in bacteria than the air of the garden. The most abundant contamination here was that of mould-fungi, like all the other localities. After a thorough examination, Hansen came to the conclusion that, without any doubt, scarcely a single one of the organisms which entered the flasks proceeded from the grains. At all events, the great abundance of bacteria in the grains does not stand in correct relation to the above-mentioned statement, which, with far greater probability, admits of the explanation that the air departs as little from the normal in this case as in others,

which contain whole contingents of organisms, but which possess a moist upper surface.

This, however, must not be misunderstood to mean that grains can be accumulated in a chosen place, and the remains after removal exposed to the weather; it is clear that this constitutes a great danger. When this residue dries and is whirled about in the air as dust, masses of bacterial germs will be carried up at the same time, and, without doubt, will constitute a source of constant bacterial contamination. For this reason places where grains have remained for any time must be washed with lime-water or chloride of lime.

In a corridor which led to the room in which the barley was turned, the flasks always received a greater contamination than anywhere else; consequently this air was very rich in bacteria.

On the malt floors the air was also characteristic; it always gave a very strong growth of mould. In the cases in question this growth consisted of *Eurotium Aspergillus glaucus*, which was otherwise rare. On the malt itself, as always, *Penicillium glaucum* occurred the most frequently.

The greatest interest, however, is connected with the examination of the different fermenting rooms, partly in Old Carlsberg, and partly in the brewery "N." In the first-mentioned rooms the air was poorer in organisms than in any of the other places examined in the whole research; in the fermenting-cellars of the brewery "N," a large number of flasks were, on the contrary, contaminated (55, 75 to 100 per cent.). The organisms which occurred in the air of this cellar were—*Saccharomyces cerevisiæ*, *S. Mycoderma*, *S. Pasteurianus*, *S. ellipsoideus*, *Torula Pasteur*, and other similar yeast forms; further, *Penicillium*, *Dematium*, *Cladosporium*, and rod bacteria. Hansen was therefore able, by a most favourable accident, to show the two contrasts in the state of the air in the most important place in every establishment connected with the fermentation industries: on the one hand, an almost germ-free air; on the other hand, an atmosphere swarming with germs. That the product of

the latter place at this time must have borne the stamp of these conditions admits of no doubt, and we find here one of the most important reasons for this, when the matter is seen with the eyes of a practical man. The air in the fermenting room may contain a world of germs which, in the fermentation industries, bring with them the most calamitous results; it is, however, possible to obtain the air free from these invisible germs, and it admits of no doubt that, on the one hand, the purification of the air in the fermenting room by passing it through a salt-water bath, and, on the other hand, the most rigidly executed order and cleanliness in the cellars of the Old Carlsberg brewery, stand in direct relation to the above-mentioned results. Hansen's results, therefore, here again contain a warning, which cannot be repeated too frequently. We will again return to these points when we discuss the same author's researches on the diseases of beer.

Quite recently Hansen has devised a method for the examination of water with respect to its use for brewing operations. The principle of the method is, that it is only necessary to know, for brewing purposes, whether the water contains such organisms as are capable of developing in wort and beer. To this end, small quantities of the water in its original or diluted state are added to a series of flasks containing sterilized wort and sterilized beer. An examination of the flasks after incubation in a thermostat at 25° C. for fourteen days will then afford information regarding the nature and number of the organisms present in the water in question.

CHAPTER III.

THE SCHIZOMYCETES, OR BACTERIA.

The more our knowledge of these small organisms becomes enlarged the more difficult it is to give to them a general definition. They are known in all forms, from the finest points or spheres to green algal-like threads, and they appear very nearly in all possible places under the most various conditions as the cause of putrefaction or decay (Saprophytes), of diseases (pathogenic forms), and of fermentation (zymogenic forms).

The first knowledge of these forms was obtained in consequence of small quantities of different substances being placed under the microscope and examined with high powers. In putrefying flesh there were found very small spherical bodies, which clearly multiplied by transverse division (micrococci, "bacterium termo"); in sour milk there occurred short rod-like bodies (bacteria); in rotting vegetable material larger spherical bodies (macrococci) and long, fine threads (bacilli and leptothrix) were found; in saliva, on the contrary, there were seen very fine, wavy, almost broken threads (spirilla, spirochæta), &c. On this account it was convenient to provisionally hold fast to these forms, and to describe them as so many distinct species; and Cohn particularly has earned credit in this respect, since to him is due the first systematic classification of bacteria.

We will next consider the forms and the individuals somewhat more closely. As before stated, the bacteria in their simplest form occur as spherical bodies of different sizes, down to bodies so minute that they can scarcely be seen with the strongest powers, and only give evidence of their existence as organisms by their multiplication through fission. Bacteria are accordingly divided into macrococci

and micrococci (Fig. 8; 1—5). From the coccus a gradual transition takes place to the short rod (bacterium, Fig. 8; 6, 7, and 9), from this to the somewhat longer rod (bacillus, Fig. 8; 8). When this is enlarged in the middle, and drawn out towards the ends, the clostridium-form (Fig. 8; 10) is produced. If the rod is so much elongated that it becomes

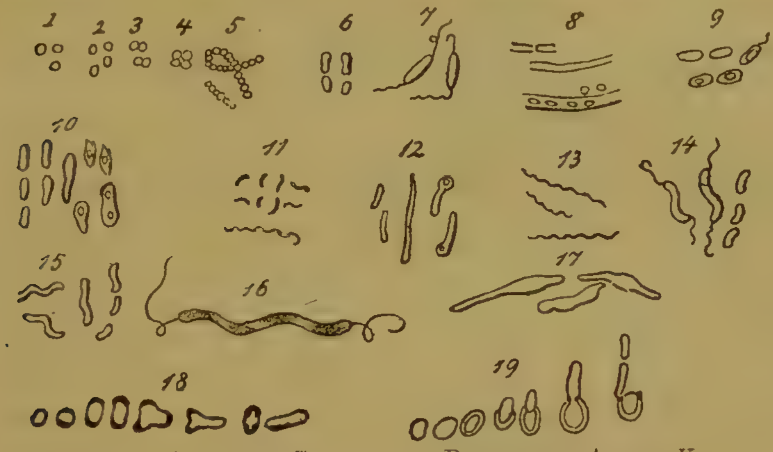


FIG. 8.—FORMS OF GROWTH OF BACTERIA. AFTER KOCH, PRAZMOWSKI, AND HUEPPE.

Micrococcus forms (spherical 1, 3, 4, 5; elliptical 2); rod forms (straight 6, 7, 8, 9; spindle-like 10); spiral forms (comma bacillus 11—16); involution forms (17); germinating spores (18, 19).

more like a long, fine, straight thread, it is called leptothrix; if it is short or long, more or less curved, broken or spiral, we have vibrio, spirillum, spirulina, or spirochæta (Fig. 8; 11—16); an entirely different form, which appears as ramified threads, is the cladothrix form. To these must be added the remarkable irregular, swollen, or curved forms which many bacteria can assume, without the cause of the alteration being sufficiently known (involution forms, Fig. 8; 17). These forms are easily found when a surface growth of the acetic acid bacterium is examined.

We will now select one of these forms, and submit it to a thorough examination with a magnification of about 1,000 diameters. Like every other cell it contains protoplasm, a homogeneous, feebly refractive mass, in which numerous small bodies can here and there be detected, especially if the

cell is not in its most active growth. Sometimes a very bright spot is found in the middle of the cell, which, in analogy to the higher plants, is considered to be a cavity or vacuole. In some particular bacteria certain solid substances can be detected in the plasma; for instance, sulphur grains in those bacteria which live in water containing sulphur. In some varieties the plasma can, at certain times, be coloured blue by iodine, from which the presence of substances resembling starch is inferred.

Surrounding this protoplasmic body we find a cell-wall or membrane. A thorough examination of this, by means of staining, will often show that this membrane in its outer layers is swollen up into a gelatinous mass; this is especially the case when whole masses of bacteria are aggregated together. From a chemical standpoint it must be provisionally considered that this cell-wall is of a different nature in different species. In some it reminds us of the cellulose of the higher plants, in others it appears rather to resemble the albuminoids in its properties.

Many bacteria contain blue, red, yellow, or green colouring matters, which produce very intense colours perceptible to the naked eye. Under the microscope, however, the individual bacteria show only a very feeble colouration. It has not yet been determined with certainty in what part of the organism the colouring matter is situated.

A remarkable property of many bacteria is their—at least apparent—free movement. This goes on either quickly or slowly whilst the bacteria vibrate or turn on their longitudinal axes, and assume open or contracted spirals. In some of these mobile forms we can observe, by high magnification, very fine cilia or flagella; in how far these must be considered as organs of locomotion is not yet determined, and whether they have their origin from the membrane or from the cell contents is quite as undecided.

The multiplication of bacteria takes place in different ways. In the main, a multiplication by division and by spore formation in the interior of the cell can be distinguished. The first mode of multiplication has been observed in detail in the

larger forms: a fine diagonal membrane appears, which little by little increases in thickness and becomes gelatinous; after this the organism separates at these diagonal membranes into smaller rods or pieces. Still, long before a trace of these membranes can be observed, the organisms when stained show that they consist of a series of segments, each of which corresponds to a member later to be formed.

It was proved by the investigation of the forms of growth of bacteria in the manner previously mentioned (most recently particularly by Zopf), that the same species of bacterium can occur in very different forms, for instance, as spirillum, leptothrix, bacillus, bacterium, and coccus, and we thus obtained the important knowledge in the history of these plants that the names quoted very often only express forms of growth of the same species, and not distinct species. The following question, however, remains to be answered: Under what conditions does a species occur in this or that particular form? Upon this point we know very little at present.

With many bacteria, moreover, multiplication by spores takes place in the following manner:—The plasma in the cell becomes darker, and often considerably granular; after that a small dark body is seen, which quickly increases in circumference, and at the same time becomes very refractive; meantime the greater portion of the plasma of the cell disappears, since it is employed for the building up of the spore; this then appears to be enclosed in a clear liquid which little by little disappears; finally, the cell-wall shrivels up, and is then only a withered appendage to the ripe spore. This organ is often known as the "resting-spore" (Dauer-spore), for two reasons, viz.: firstly, because these spores actually possess a greater durability and resistance to outside influences than the vegetative rods; and, secondly, because the spore formation, as a rule, only takes place when the food-stuff of the culture is either exhausted or unfavourable to the further vegetative growth of the organism; the spores, therefore, serve to preserve the life of the organism during this critical period.

As soon as favourable conditions of nutriment and tempera-

ture again appear, the spores germinate. They first increase in size, and the contents lose their strong refractive power. A bacterium then grows out from the spore, and after a time the wall of the spore is seen to burst and divide into two lids (compare Fig. 8; 18 and 19). The emerged rod then multiplies in the usual manner.

The spore-forming bacteria are now generally divided into two classes, distinguished by the formation of *endospores* in the one, and of *arthrospores* in the other; of these the first-named form their spores in the interior of already developed rods; the last mentioned have, however, in the investigations hitherto made, shown no such interior spore-formation. They are undifferentiated elements of a chain of vegetative cells, which become the starting points of new generations of growing cells (for instance, *Leuconostoc*, *Mycoderma aceti*). Perhaps by continued investigation endogenous spores may also be found in all species of the last-mentioned division; it is only a supposition that the above-mentioned elements in a chain of organisms must be considered as corresponding to the spores.

Finally, in the study of forms of the bacteria, we must mention the so-called zooglœa formation. In all branches of the fermentation industries, it is known that in places where the cleaning is not sufficiently looked after, slimy, fatty masses may occur, which increase little by little in thickness. The cause of this is generally a growth of bacteria under such conditions that the single cells lie very close to each other, and at the same time greatly swell at the outer gelatinous layer of the cell-wall; during the continued growth the slimy layer also increases in thickness, and often at the same time assumes a certain characteristic form. Such slimy masses—known in the sugar manufacture as "frogs' spawn"—occur on solid as well as in liquid materials.

Pasteur distinguishes, from a biological standpoint, two classes of micro-organisms; the *aërobian*, which require oxygen for their life, and the *anaërobian*, which can dispense with this gas. The latter are promoters of fermentation; they are also able to live very freely in the presence and

under the influence of oxygen; they are then no longer promoters of fermentation, and in this condition rank as aërobian; if they are then again forced to exist with insufficient oxygen, they become promoters of fermentation, since they can perform chemical work and decomposition without the influence of oxygen ("Compt. Rendu," lxxx., 455). During the course of the present year, however, observations and experiments have been made by a reliable worker, which show that Pasteur's conclusions concerning the part played by oxygen on the life of the micro-organisms is at least partly incorrect. His theories therefore give no generally correct explanation of the phenomenon of fermentation.

We will now pass in review the more important of the species, which are of especial interest in the fermentation industries.

1. The butyric acid ferment (*Clostridium butyricum*), which appears to be very widely distributed in nature, always appears in saccharine mashes, and may, if these are maintained for a lengthened period at a certain temperature, grow very rapidly and exercise a retarding influence on the alcoholic ferment. The ferment is known in the shape of short and long threads and rods, which may be either straight or curved; before the formation of spores the rods swell and form—as shown in Fig. 9, *B*—peculiar spindle-, and citron-like, elliptical, or club-like forms; at the same time the important fact of their being coloured blue by iodine appears. On germination the spores burst their outer envelope, and the germinating rod grows in the same direction as the longitudinal axis of the spore. According to Pasteur's experiments, the butyric acid ferment can perform its functions without having access to the free oxygen of the air. The ferment is capable of decomposing starch and cellulose. *Clostridium butyricum* grows most readily at a temperature of about 40° C., and is then especially able to predominate in a saccharine solution if the lactic acid ferment has already converted a portion of the sugar into lactic acid. For the same reason it occurs very much during the storage and ripening of cheese, and then contributes to give this its

pleasant odour and taste. According to Fitz the spores can

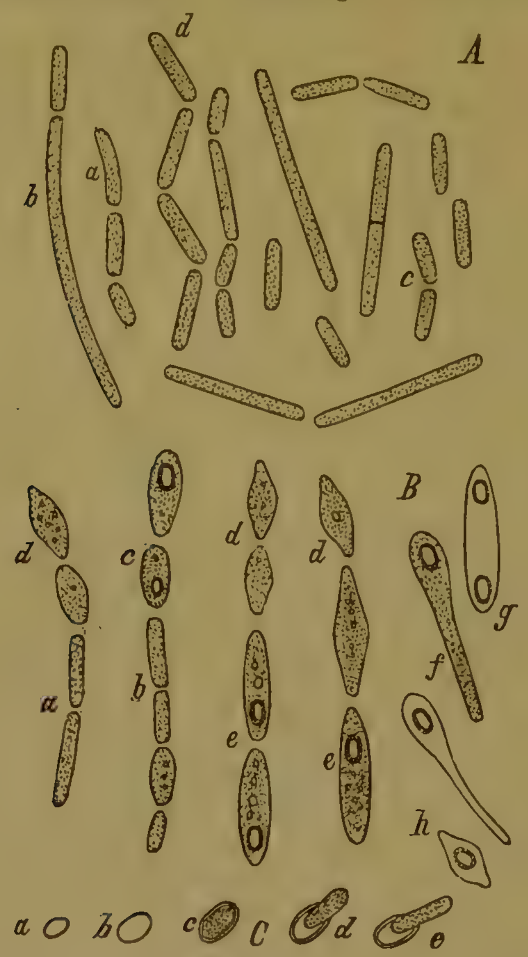


FIG. 9.—CLOSTRIDIUM BUTYRICUM. PRAZMOWSKI.

A. Active state of growth; *c*, short rods; *d*, long rods; *a* and *b*, vibrionic-like curved rods and threads. *B.* Spore formation; *b*, *d*, short rods before, *c*, *e*, during, *f*, *g*, *h*, after spore formation; *e*, from elliptical, *d* and *h*, from citron-like, *c* and *g*, from spindle-like, and *f*, from tadpole-like forms; *a*, rods which are still in active growth. *C.* Germination of the spores; the spore *a* swells to *b*, which then shows the differentiation of the membrane in exo- and endospore *c*. From the split end of the spore the contents surrounded by the endospore emerges in the form of a short rod, *d*, which is already elongated in *e* (after Prazmowski).

withstand a boiling temperature for a period of time, which is naturally here, as always, dependent on their condition

and on the nature of the substance ; Fitz gives three to twenty minutes as the limits. They can, however, also be killed by a lower temperature, if it is continued long enough ; thus they are killed by being heated for six hours at 90° C. in a solution of dextrose, but in glycerine, at the same temperature, only after six to eleven hours. If butyric acid occurs in beer to any extent, a very unpleasant taste is the result.

Grüber found associated under the name of *Clostridium butyricum* three well differentiated species, of which two are exclusively anaërobic. The first of these last-mentioned species consists of straight or slightly curved rods, which become spindle- or barrel-shaped during the formation of spores ; the second species consists of strongly curved vegetative rods, in which the spores are formed at the end. The first species forms colonies on nutritive gelatine, which are blackish-brown to black by reflected light ; the other gives yellow to yellowish-brown colonies. The third species is also capable of growth and of causing fermentation in the absence of oxygen ; its development is, however, decidedly promoted by the presence of oxygen, and it is only then able to produce spores. The growing rods are cylindrical ; with the formation of spores the rods become spindle-shaped, and in the centre of the spindle the large spore is formed. The colonies on nutritive gelatine are of a yellow colour.

It holds good for the butyric acid fermentation as for the lactic acid fermentation about to be described, that it is not produced exclusively by one species. When butyric acid fermentation occurs in distilleries, breweries, or pressed yeast manufactories, there are certainly found in many cases entirely different bacteria from those described under the name of *Clostridium butyricum.*

2. The different species of bacteria which cause lactic acid fermentation are not yet accurately known. Pasteur, in "Études sur la Bière," figures in the first plate an organism which produces lactic acid fermentation and occurs as a short rod bacterium and micrococcus. Later, Hueppe found a bacterium in a spontaneous lactic acid fermentation which converts milk-sugar and other saccharoses into lactic acid

with the simultaneous formation of carbonic acid. It consists of short, plump cells, forming endogenous terminal spores. In saliva and the mucus of teeth he found two species of micrococcus, which also possessed the power of forming lactic acid from sugar. Among the pigment-forming bacteria there are also found species which, in addition to their pigment-fermentation, are able to form from the sugar in milk so much lactic acid that the casein of the milk becomes curdled in a gelatinous form; to these belong, according to Hueppe, the celebrated red *Micrococcus prodigiosus*, and, according to Krause, a pathogenic form, the micrococcus of osteo-myelitis. Delbrück found that in a mash prepared from dry malt and water lactic acid was first formed at a temperature of about 50° C., and from this he draws the conclusion that in this case the active lactic acid ferment has its maximum temperature at this degree of heat.

The lactic acid fermentation occurs in the brewery both in the malt, in the wort, and in the after fermentation. In the Belgian beer prepared by spontaneous fermentation lactic acid is formed in large quantity, and consequently the beer possesses a sharp taste. In modern low-fermentation breweries it is endeavoured to keep both the lactic acid ferment, as well as bacteria in general, out of the fermentations. "In distilleries," says Maercker, "they are still provisionally regarded as a necessary evil. The production of lactic acid follows the yeast dressing, and its value appears to reduce itself to preventing the development of bacteria, and in this way rendering possible the pure fermentation of the alcoholic yeast."

3. The acetic acid ferment (*Mycoderma aceti* and *Mycoderma Pasteurianum*) was first thoroughly described from a morphological standpoint by Hansen; the correctness of these investigations was afterwards confirmed by Zopf, de Bary, and A. J. Brown. In "Études sur le Vinaigre," published in 1868, Pasteur gave an account of his experiments with the acetic acid bacterium, and also a method, based on the results, for the manufacture of vinegar. This method is, however, not founded on the employment of a definite selected species.

Whilst Pasteur, in the above-mentioned work, does not explicitly maintain the theory that this organism is the physiological cause of the oxidation of alcohol to acetic acid, yet Adolf Mayer expresses this opinion; and Hansen emphasizes as a certainty the fact that the formation of acetic acid is commonly effected by the action of this organism. By placing lager beer in a thermostat at 30° to 34° C., Hansen obtained a vigorous film-growth of the acetic acid bacterium. This consisted of long chains of hour-glass-like individuals, partly as bacterium and bacillus forms, partly as curved forms.

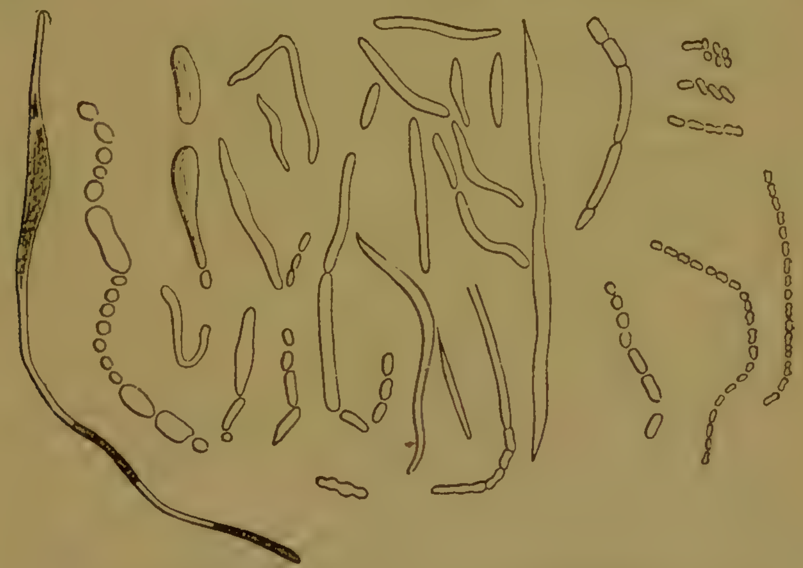


FIG. 10.—MYCODERMA ACETI AND MYCODERMA PASTEURIANUM. AFTER HANSEN.

The frequent and very rapid formation of differently shaped, irregularly swollen forms is peculiar to *Mycoderma aceti;* with other bacteria these forms do not occur until a very advanced stage, and then, in all probability, stand in close relation to a deficient supply of nutriment. This, however, cannot be the case with our organism. We have here one of the first bacteria, regarding which it has been shown that the same species can occur in very different shapes. By means of his staining experiments with *Mycoderma aceti*, Hansen discovered that two distinct species are hidden under this

name, of which the one—like most other bacteria—is stained yellow by iodine, whilst the other exhibits a blue colouration with the same reagent. In all other respects they behave alike. A fact of interest is the observation made by Hansen that a pure culture of the two species of *Mycoderma* in beer does not exercise any influence on the colour or brightness of the liquid. It is therefore possible, to a certain extent, to ascertain whether or not these organisms are alone present, since other bacteria when present in beer bring about a turbidity of the liquid. In order to be able to develop with vigour, *Mycoderma aceti* not only requires a very plentiful supply of free oxygen, but also a fairly high temperature. Hansen found that a temperature of about 33° C. was the most favourable when Carlsberg lager beer was used. In a well-conducted store-cellar (1° to 3° C.) there is therefore no ground to fear *Mycoderma aceti*. As soon, however, as the beer leaves the cellar, and is stored at a higher temperature, there is always danger.

Pasteur has stated that, by the oxidation of alcoholic liquids, ethyl alcohol is converted into acetic acid, and by continued oxidation the latter is further converted into carbonic acid and water. This has been recently confirmed by A. J. Brown, whom we have to thank for the most complete chemical analysis in this direction.

In France, indeed, the method founded on Pasteur's theory is employed on a large scale for the production of vinegar; the manufactories so constructed work, according to Wurm, seven to twelve times as quickly as those constructed in accordance with the ordinary old method.

According to the communications of Hansen, there are many species of bacteria commonly occurring in beer which secrete invertive ferments. Within these species we again find a group which develops an invertive activity in a pure saccharose solution; this, however, is stopped when yeast-water is added. Similar features were observed by Wortmann with those bacteria which secrete diastatic ferments. The formation of chemical soluble ferments is generally very widely distributed in the bacterial world, and in this we find

one of the means by which these minute organisms develop such an immense chemical activity in the economy of nature.

4. In addition to the above-mentioned species of bacteria, there also occurs in fermenting liquids a number of other forms, the life histories of which are only very incompletely known. Both in bottom-fermentation and in top-fermentation (especially in distilleries and pressed-yeast manufactories) there occurs a micrococcus, the injurious action of which is strongly emphasized in the Journals of these industries. In spirit mashes this organism occurs both singly, and also in dumb-bells and pearl-like strings; in bottom-fermentation lager beer, it appears as more or less spherical, water-grey, small bodies, generally arranged in groups of fours; they were described by Hansen under the name of *Sarcina* (Fig. 11). Of these latter we know that they are both able to render the liquid turbid, and also to give it an unpleasantly sour and bitter



FIG. 11.—SARCINA.

taste; further, that they only develop very slowly, when the temperature of the store-cellar is kept below 5° to 6° C.

Reincke has, according to papers published in the "Wochenschrift für Brauerei," often had occasion to examine both bottom- and top-fermentation beers, which were attacked by Sarcina. He states that lager beer, so attacked, quickly throws down a considerable sediment, and develops a bad taste and smell. The well-known Berlin white beer often assumes a red colour, and in that case is always infected with Sarcina. The growth increases considerably after a few days at a somewhat higher temperature—temperatures between 10° and 14° C. appear to be particularly favourable. The author correctly lays stress on the fact that it is not certain whether Sarcina or the rod bacteria, which are also present, are the actual cause of the calamity; it is only known that in red beer, for instance, the presence of Sarcina is characteristic of abnormal

conditions. Whether this is the cause or the result can only be determined by exact investigations.

Lindner has recently found, by means of pure cultures by Koch's method, a yellow, white, and orange Sarcina in beers in addition to the so-called "beer sarcina." The multiplication of these forms is favoured by neutral or alkaline nutritive media. Only the white and orange forms develop in acid substrata. Quite recently Lindner has increased our knowledge of this organism by the issue of an elaborate report from the Berlin Brewing Institute. He describes several species of Sarcina, some of which show only a two-dimensional growth, whilst others exhibit a three-dimensional growth also. The most important of the former class he calls *Pediococcus cerevisiæ*; this species readily develops in beer at a temperature of 12·5—20° C., it produces a turbidity in beer, and was usually found associated with ropiness, although all attempts to inoculate the organism into sterilized beer failed. It occurs very frequently in the air of breweries and elsewhere. Other species described by Lindner were *Pediococcus acidi lactici*, *P. albus*, *P. candida*, *P. maxima*, &c.; they all produce acid, and are quickly killed at a temperature of 60° C.

In the fresh residues from spirit mashes, which are employed as fodder, Bräutigam found a sarcina-like micrococcus, which possesses pathogenic properties.

The characteristics of the species of micrococci, which occur here in different sizes, are not known. It is, therefore, at present impossible to answer questions on their conditions of existence and their occurrence in other places. As is known, there are not a few micrococci which cannot be morphologically distinguished with certainty, yet which possess in the highest degree different pathogenic properties.

5. In consequence of the investigations carried out by Laurent, it appears that the most essential active factor in the fermentation of black or rye-bread is not, as was formerly supposed, a species of yeast (*Saccharomyces minor*, Engel), but a bacterial form, which he has named *Bacillus panificans*. (In the preparation of white bread, however, the alcoholic yeast, Saccharomyces, is, as formerly, to be considered the

active ferment.) When he cultivated this bacterium in a pure state, and added such a culture to the bread-dough, exactly the same phenomena were observed as when the ordinary sour dough was employed. In cultures on gelatine this bacterium gives rise to pale chrome-yellow colonies with rounded edges, which are greyish-brown by reflected light; the growth of the colonies takes place very slowly, and they do not liquefy the gelatine. If a diluted drop is brought with a needle to a reagent-glass containing nutritive gelatine, a series of colonies is formed in the gelatine, and a large colony is slowly developed on the surface, which is impressed on the sides like a section of a fern-leaf. The bacillus develops both in the presence of ordinary air and also without free oxygen; it has its most favourable temperature at 33° to 34° C., but it is able to grow at temperatures between 6° and 45° C. In the first days of the culture it occurs as short, mobile rods, later as long bacilli, which produce a film on the nutritive liquid and form spores; these are only killed after boiling for ten minutes at 100° C. The bacillus easily dissolves the gluten substance of the dough, grows in starch-paste and in mixtures of saccharose and mineral substances. It is found in an active state in large quantities in bread, and, according to the author's researches, it can withstand for twenty hours the action of an artificial gastric juice. In the excreta it is found still more abundantly, and it appears to be generally distributed in plants and in various substances.

During the past year Dünnenberger has published the results of a series of experimental researches which have led him to an opposite conclusion. He found that the normal bread-fermentation is an alcoholic one, whether yeast, rennet, or sour dough be used for panification. According to this author the budding-fungi must be considered to be the only important fermentative organisms, and for the normal bread-fermentation bacteria are an unnecessary impurity and absolutely surperfluous. The rising of the dough is, in the first place, caused by the carbonic acid liberated by the alcoholic fermentation. Afterwards, the expansion of air, and conversion into gas of alcohol and of water by the heat of the baker's oven,

also assists in the process. The formation of volatile fatty acids by bacteria also helps in an accessory and subordinate manner.

6. A phenomenon, which has hitherto been only partially explained, is the so-called viscous fermentation, which, for example, takes place when sugar-containing juices are separated from parts of plants. Micro-organisms are always found in this formation of viscous substance, which can be again produced when the organisms are introduced into new vegetable sap. Pasteur has also figured in his "Études sur la Bière," on Plate I., Fig. 4, bead-like chains of spherical organisms, which make wine, beer, and wort capable of being drawn out in threads; they seldom occur in wort, still more seldom in beer. (Compare Fig. 12.) There are also found in such viscous liquids irregular cells of considerable size, which are not yet more closely known. During the development of the viscosity carbonic acid, and often also mannite, is formed. According to all analogy this viscosity must be considered as a phenomenon nearly related to the commonly occurring zooglœa formation of certain bacteria; it is, however, also regarded as a product of the decomposition of sugar.

7. The so-called "Kephir," on which the investigations of Kern have thrown some light, is a sparkling, alcoholic, and acid milk, which is prepared by the inhabitants of the Caucasus from cow's, goat's, or sheep's milk. It is prepared by adding a peculiar ferment, "kephir-grains," to milk. These are white, irregularly shaped, uneven grains, about the size of a walnut and of tough gelatinous consistency, and when dried become cartilaginous and brittle. The most essential part of these grains consists of rod-like bacteria which are connected in threads and have developed viscous-like membranes. Kern calls these *Dispora Caucasica*. There is also found in kephir-grains a yeast-like mould. In the preparation of kephir a little milk is first poured on the grains and allowed to stand for twenty-four hours, the milk is then poured off, and the grains preserved for future use. This milk is now mixed with fresh milk, and poured into

bottles which are corked, or into leathern sacks which are tied; after some days a fermentation has taken place. It now contains about 2 per cent. of alcohol. This result is probably brought about equally by the above-mentioned *Dispora* and the yeast-like cells in combination with the lactic acid ferment which is probably always present in milk. The last-mentioned converts a portion of the milk-sugar into lactic acid; the alcohol and a part of the carbonic acid result from the yeast-like cells, since these are able to bring about a feeble alcoholic fermentation in glucose solutions. Then as the fermented milk contains a considerably smaller quantity of coagulable caseïn than the ordinary sour milk, it may further be assumed that the above-mentioned *Dispora* is also able to partly liquefy (peptonize) the coagulable caseïn, perhaps even with the help of the gelatinous masses secreted by the bacteria which are found in the kephir-grains, but are not present in the fermented milk. If the above-mentioned kephir-grains are allowed to remain in milk, they grow very slowly, and only attain, according to the researches of de Bary, a double size after the lapse of several weeks; this author considers it probable that under such conditions single *Dispora* cells separate themselves and give rise to new kephir-grains.

According to the mode of preparation published by A. Levy, kephir can also be obtained without the addition of any special ferment. When milk which has become sour is violently shaken an effervescing alcoholic kephir-like drink is obtained, which, as regards taste, &c., cannot perceptibly be distinguished from kephir prepared with kephir-grains; according to de Bary's experiments the kephir obtained by shaking contains 1 per cent. by volume of alcohol, whilst a sample of the ordinary kephir contains 0·4 per cent. by volume.

8. The so-called "Dextran ferment," *Leuconostoc mesenterioides* (Fig. 12) occurs spontaneously in beetroot sap and in the molasses from the manufacture of sugar, where it forms large, slimy masses (frogs' spawn), and increases with great rapidity. According to Van Tieghem, the organism is able to secrete a ferment that inverts cane sugar, which it

then uses for its nutriment; and since it increases so rapidly it can in a short time consume considerable quantities of sugar. The slimy mass (dextran) formed by the ferment is as clear as glass and encloses within it the pearl-like chains of the ferment.



FIG. 12.—LEUCONOSTOC MESENTERIOIDES. AFTER VAN TIEGHEM AND CIENKOWSKI.

1, spores; 2, spores after germination, with strongly gelatinous membrane; 3, 4, 5, 6, successive stages of the division of the micrococci and gelatinization up to the curved forms; 5, 6, 7, a conglomeration of small zooglœa masses; 8, section through an old stage of a compound zooglœa with numerous long torula-like threads; 9, chains of cocci, interrupted by single spores, which can be distinguished from the cocci by their larger size.

9. We spoke above of the hay-bacillus (*Bacillus subtilis*) as one of the most frequently occurring bacteria, both in the air and on vegetable life; we must now mention one of the bacterial forms most frequently present in water. This is the *Crenothrix Kühniana*, or pest of wells (Fig. 13).

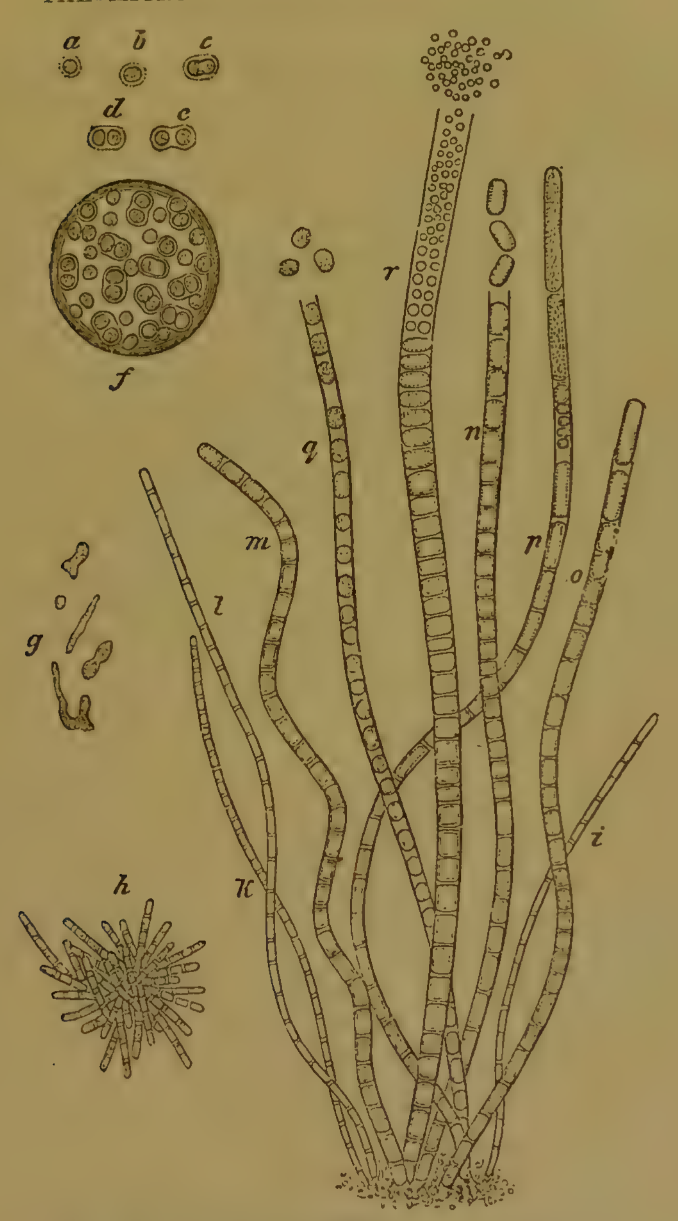


FIG. 13.—CRENOTHRIX KÜHNIANA. AFTER ZOPF.
a—e, cocci in different stages of division ; *f*, small, round cocci-zoogloea ; *g*, (natural size) zoogloea ; *h*, colony of short filaments composed of rod-like cells, originating from the germination of a small collection of cocci ; *i—r*, filaments, partly straight, partly curved spirals (*l*, *m*), of very varying thickness, with more or less pronounced contrast between the base and apex, and different stages of the division of their limbs and sheaths. The sheathed filament *r* shows short rods at the base, which are more divided above into small cylindrical pieces. At the apex are seen the cocci arising from the longitudinal division of the cylindrical discs.

This ferment, frequently associated with *Beggiatoa alba*, occurs in every water which contains organic matter; sometimes it multiplies to such an extent that it may make the water unfit for use. Thus, according to Zopf, great calamities have been caused by this ferment in the water supplies of Berlin, Lille, and certain Russian towns. In consequence of its power of storing iron compounds in its walls, it forms red or brown flocks in water. Its forms are very beautiful; it occurs in the form of cocci, which by partition and formation of viscous matter form zooglœa; these cocci increase to filaments, which articulate and are seen with distinct sheaths; they increase in thickness towards the apex; when they have arrived at a certain age, they divide within the sheath into smaller pieces, which become round and issue forth as rods, macro-, or micrococci; these are able to float about in water. We do not yet possess a more exact knowledge of the life history of this beautiful bacterium.

CHAPTER IV.

THE HYPHOMYCETES, OR MOULD-FUNGI.

The mould-fungi ordinarily encroach upon the fermentation industries in a somewhat different manner from the bacteria. Whilst the latter—in distilleries as a rule, in breweries only exceptionally—make their appearance with great power during the fermentation, and are therefore able to bring about important changes in the course and in the products of the fermentation, the mould-fungi, on the contrary, usually occur outside the true field of the fermentation, since they select as places of growth the vessels, tools, rooms, the green malt, and the quiescent masses of yeast. Accordingly the mould-fungi have a more subordinate, but nevertheless very real, importance. If we only sufficiently examine a growth of mould which has developed on the roof or walls of a fermenting room, or on the sides of a vessel, it will very soon be found that we have practically never to do with a mould growth alone; in nearly every case bacteria and yeast-like cells are found under the filaments of mould. These filaments extend upwards, and thereby raise the foreign elements, which in this exposed position are more easily removed, partly by the workmen, and partly by the air. There are present during malting, on the raw materials containing starch, all sorts of microscopic organisms. The mould-fungi are usually regarded as the most dangerous enemies, and this is certainly due to the fact that they are visible to the naked eye during development, and thus obtrude themselves upon our notice in an unmistakable manner. If, however, the numerical superiority be taken as decisive, then the bacteria, which are always present in large quantities on green malt, must certainly be placed in the

front rank. Judged from this side, it may even be considered as doubtful whether the greatest influence on the product must be ascribed to the mould-fungi (*Penicillium*, *Aspergillus*, &c. &c.), when these are met with in vigorous development on the malt, or whether it is not far more probable that it is the numerous other organisms accompanying them which here play the most important *rôle*.

I have often found on the surface of pieces of pressed yeast a fine white parasitic growth, which most frequently consists of a mould mycelium, belonging principally to forms resembling *Chalara* and *Dematium*. It is very possible that these plants, when they form a thick layer on the surface of masses of yeast, retain by their respiration a portion of the free oxygen which is necessary in order to enable the quiescent yeast to remain alive for a longer time. Under these conditions I always, without exception, also found bacterial growths.

The truth is, acccording to observations made in breweries and elsewhere, that a mould growth nearly always serves as an indicator that other organisms of a doubtless more injurious and powerful character are present in the growth. It is, therefore, of great importance that the walls of the fermenting room should be smooth; this is obtained with the greatest certainty by employing the enamel paint now so much in use.

The following consists of a review of the most important mould-forms, which are of interest for the fermentation industries.

1. *Botrytis cinerea* (Fig. 14), which forms small greyish-yellow patches on moist, decaying vegetable matter, and can even occur on wort, is one of the most beautiful of the moulds to be here mentioned. From the greyish-brown mycelium, the conidia-carriers are thrown up; these are perpendicular, articulated filaments, generally arranged in tufts. They grow to the height of 1 mm., after which the apical cell throws out near its point, and almost at right angles, two to six small branches (C''). The lowest of these branchings are the longest; they separate again at their

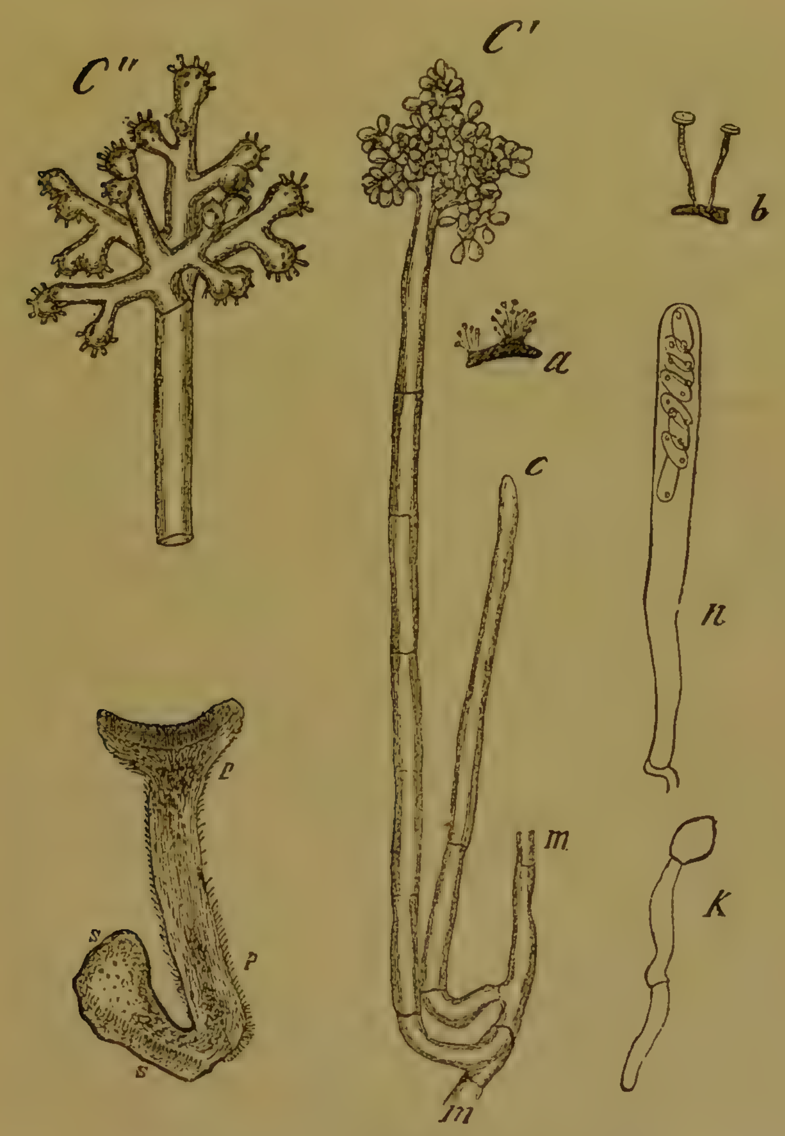


FIG. 14.—BOTRYTIS CINEREA. AFTER DE BARY.

a, *b* (natural size), Sclerotium, from which at *a* the conidia-carriers, at *b* the fruit-envelopes, are thrown up ; *c*, *C'*, conidia-carriers (*C'* with conidia just ripe) springing from the mycelium thread *m* (magnification about 200) ; *C''*, end of a conidia-carrier, with the first commencement of the formation of chaplets of conidia from the branchings ; *k*, germinating conidium (× 300) ; *p s* (slightly magnified), section through a sclerotium *s*, from which a very small fruit envelope (*p p'*) is thrown up ; *n* (× 300), single spore envelope with eight ripe spores.

ends into one or more short side branches. The topmost branches are almost as wide as long. Subsequently there arises a system of branchings which is shaped like a cluster of flowers or a bunch of grapes. When the longitudinal growth is at an end, the branches separate their inner space from the main stem by a transverse wall close to the latter. At the same time the ends of the branches and of the main stem swell very much, and on the upper half of each swelling now appear together several small spots, which quickly increase to oval blisters, filled with plasma, and decreasing, stalk-like, to their base. When these conidia (*C'*) are completely developed, the walls of the branches carrying them become shrivelled up, and the conidia are consequently brought so closely together that they form a loose, irregular aggregation which easily falls off. If these clusters are placed in water, the conidia loose themselves from their stalks, and the envelopes of the branches, devoid of plasma, shrivel up or are only to be found in traces; their former place of attachment to the main thread appears only as a slightly raised scar. The member next below can now throw on one side the shrivelled apex, grow upwards, and form a new cluster; this can be repeated several times, whereby the conidia-carriers attain a considerable height.

Under certain conditions this mould can assume a peculiar state of rest, the so-called sclerotium (*scleros* = hard), *a*, *b*, *p s*. The mycelial threads branch extremely freely, and the branches intertwine themselves into an uninterrupted body of diverse shape, circular to narrow spindle-shape, and of varying size up to a few lines; the extreme ends of the filaments are brown to black, and the ripe, solid sclerotium thus consists of an outer black skin and an inner colourless tissue. Such bodies are also capable, after a long period of rest—at least one year,—of forming a new growth, and may even be compared with the bulbs and roots of the higher plants. If the sclerotium is brought into a moist place soon after it comes to maturity, the inner colourless branches break through the black outer skin and throw up

the conidia-carriers (*a*). If, however, the sclerotium is not placed in a moist place until after it has been in rest for some time, there develops from the inner tissue a large tuft of filaments, which shoot up perpendicularly and finally spread out to a flat plate-shaped disc (*b*, *p s*); the ends of the filaments appear parallel on the free upper surface of the disc; some of these remain thin, others swell up to club-shaped filaments, and each of these filaments forms in its interior eight oval spores (*n*). The mould has now entered upon the fruit-bearing stage. The spores germinate when they are set free, and the germinating threads grow into conidia-carriers.

According to Bersch, Fitz, and Reess, this plant is the cause of one of the diseases of wine, which shows itself as an unpleasant smoky taste and smell. Cases of similar disease have been occasionally observed in breweries; it has, however, not yet been determined with certainty whether they are caused by this mould.

2. A mould, which enjoys a very wide distribution in the fermentation industries, especially on green malt, is *Penicillium glaucum*. It forms a felt-like mass on the substratum; it is at first white, then grey or bluish-grey, and it spreads with great rapidity. The mycelium consists of transparent branched and divided threads, which, when they are immersed in liquids, are able to swell somewhat irregularly. From these threads the conidia-carriers (*A*, Fig. 15) are thrown up perpendicularly. They consist of elongated cylindrical cells, the terminal cell of which soon stops in its longitudinal growth, and assumes a spike-like form; the cells next below throw out one or more opposite branches, which rise up close to the terminal cell, and, like this, consist of one spike-like cell. In more vigorous examples, the branches may again shoot out (compare Fig. 15, *A*), or there may also proceed from the next cells similar branches, which shoot out, and become pointed as described above. In this tuft of branches each pointed cell (sterigma) breaks up into a series of spherical conidia, and finally, the tuft carries a large number of conidia, arranged in series, which, when they are

ripe, are readily scattered. These round, smooth conidia give to the patches of mould the well-known greyish-blue colour; when they are sown on moist substrata they are able to germinate at once. In culture experiments with this plant,



FIG. 15.—PENICILLIUM GLAUCUM. AFTER BREFELD.

A, Conidia-carrier; *B*, organs of generation; *C*, structure of the growing body (*a*, the further developing carpogonium; *b*, sterile threads); *D*, very young, growing body in section (*a*, ascogeneous hyphæ; *b*, sterile portion of the growing body; *m*, mycelium); *E* and *F*, ascogeneous hyphæ (*a*), with young tube structure (*s*), and sterile mycelial threads (*m*) from a more developed growing body; *G*, group of tubes with spores; *H*, spore; *J*, germinating spore; *K*, young mycelium (with spore at *x*).

Brefeld made the interesting observation that *Penicillium* can occur under certain conditions with an entirely different form of growth; he enclosed cultures of the mould-fungus on slices of coarse, non-acidified bread between glass plates, and allowed the culture to further develop with the greatest

possible exclusion of the atmospheric air. There appeared then in couples on the mycelium short thick branchings, which twisted together (*B*) ; the one part of this spiral commenced to throw out short filaments (*C*), whilst the mycelial thread carrying the spiral gave rise to numerous fine branchings, which enveloped the spiral and formed a case (*D*), possessing an inner solid and an outer felt-like layer; little by little the inner cells became coloured yellow, and the outer loose cells were thrust off. In this small yellow ball a formation then gradually took place by the continued branching of the above-mentioned spiral filaments of swollen cells (*E*, *F*, and *G*), in each of which were eight spores. These were large and lenticular, and furnished on the margin with a circular furrow, and on the outer membrane (exosporium) with three or four slight ridges. After the falling together and absorption of all the remaining interior elements the spores were at last free, and the small yellow ball was then filled with the powdery spores. The entire development requires six to eight weeks. The fertilized spores may be preserved for many years without losing their power of germination. When the spores are sown (*H*) the exosporium bursts open like a valve at the circular furrow, and the endosporium swells and emerges (*J*), and elongates itself to a germinating filament, which quickly gives rise to the conidia-carriers.

Penicillium possesses the power of secreting an invertive ferment, which is able to convert saccharose into other sugars.

3. *Eurotium Aspergillus glaucus* (Fig. 16), whose development was first thoroughly described by the celebrated de Bary, forms a fine felty greyish or greyish-green covering on various materials, and is able to grow with the greatest luxuriance on green malt.

The mycelium consists, as with *Penicillium*, of fine transparent and branched threads, provided with transverse septa. Some of the hyphæ are thrown up perpendicularly, are thicker than the remainder, and are very occasionally branched or divided by septa. Their upper ends swell to

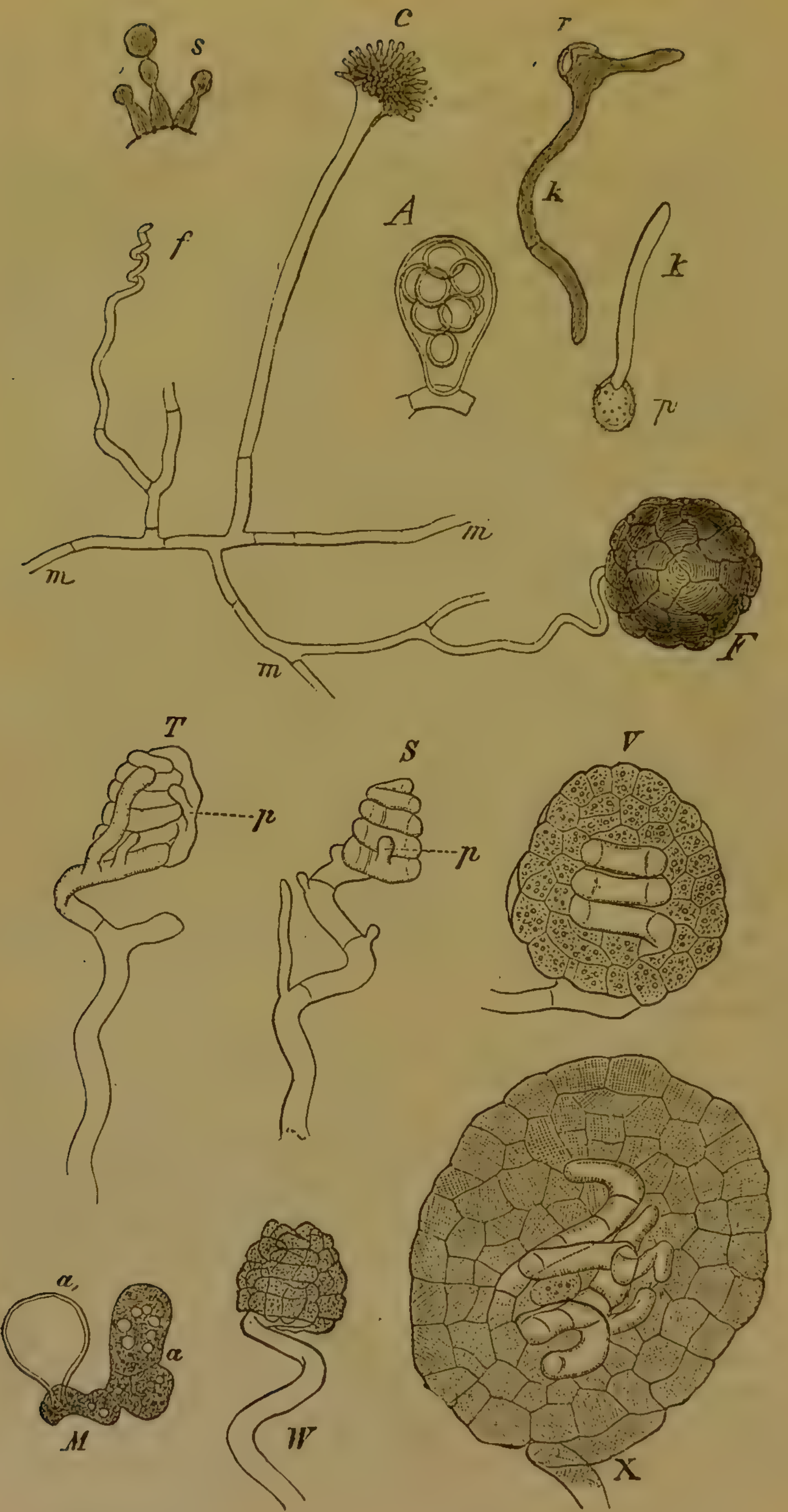


Fig. 16.—Eurotium Aspergillus glaucus. After De Bary.

spherical flask-shaped heads (*c*), and these throw out from their entire upper portion radially divergent filaments of cylindrical form; these sterigmata then throw out at their apex small round excrescences, which are attached to the sterigmata by greatly contracted bases, and after some time are defined from the former as self-dependent cells (spores, or conidia). After the formation of the first spore, a second begins to form on the crown of the sterigma, and pushes the first up; a third then forms, and so on. Each sterigma thus carries a chaplet of spores, of which the youngest occurs closest to the sterigma. This happens at the same time over the whole surface of the swollen ends of the conidia-carrier, which is then finally covered with a thick head of radially arranged chaplets of spores; these masses of spores form the greyish-green dust which covers the masses of hyphæ. Finally, the spores separate from one another; they have then a fine wart-like appearance on their outer surface. These small bodies are able at once to germinate (*p*) after their separation, and quickly develop a new mould-fungus—on this fact depends the rapidity with which the plant spreads. Under certain conditions, which are not yet sufficiently known, but which in every case appear to be connected with a free supply of nutriment, the mould develops fruit-spores. These take their beginning as tender branches, which, at the termination of their vertical growth, begin to twine their

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Description of Fig. 16.

m, *m*, Mycelial threads, carrying a conidia-carrier, *c* (from which the conidia have fallen), a ball-fruit *F*, and the first rudiments of a carpogonium, *f* (× 190); *s*, three sterigmata from the crown of a conidia-carrier, showing the conidia detached; *p*, germinating conidium (× 250-300); *A*, spore sheath, or germinating spore; *k*, germ filament; *S*, spiral carpogonium, at *p* the commencement of the growth of the encircling pollinodia; *T*, older stage; *W*, carpogonium, already surrounded by the envelope; *V*, longitudinal section of an older stage, in the centre the carpogonium, surrounded by the envelope in many layers; *X*, longitudinal section of a later stage of the development of a carpogonium, enveloped in a sheath of many layers (its convolutions are loosened, and it commences to throw out the ascus-forming branches); *M*, portion of an older ascus-bearing branch; *a*, a young ascus; *a′*, an older burst ascus.

free ends like a corkscrew (*f*); the spirals gradually become less separated, until finally they are so closely in contact that the entire end of the filament takes the form of a helix (the carpogonium). There then grow from the lowest spiral of the helix two or more small branches which are closely joined to the spiral. One of these small branchings (*S*, *T*, *p*), the so-called pollinodium, quickly outstrips the other in growth, and its upper extremity reaches the apex of the helix and becomes fused with it. This and the other branches now throw out new branchings, which surround the spiral, and gradually become so interlaced that finally the spiral is surrounded by an unbroken envelope (*W*). These branchings divide by septa perpendicular to the surface, and the envelope consequently consists of short, angular cells, in which new septa, parallel to the surface, appear, so that the envelope is many-layered and thick (*V*, *X*, *F*). The small sphere now formed is about $\frac{1}{4}$ mm. large, the outermost layer is yellow, whilst the inner layers remain soft, and later are dissolved. The spiral after a time extends, and throws out on all sides branched filaments, which dislodge the interior layers of the envelope. These branchings finally take the form of a sac (*M* and *A*), and in each eight spores are formed. After the breaking up of the spore-sac, the spores lie loose in the interior of the fruit, and attain their freedom by the rupture of the now fragile walls of the latter. The spores are, as with *Penicillium*, biconvex, wart-like, and possess, besides an outer stout membrane, an inner one, which, on germination, bursts the outer part into two valves (*r*). In addition to this variety, several others, closely related, occur in nature, and also find their way to the places mentioned here. In the greater number of these species only the conidia stage is known.

4. In the preparation of the strong, fermented rice wine, saké, the so-called *Aspergillus Oryzæ* is systematically employed. It has been investigated by Ahlburg, Atkinson, Cohn, and Büsgen. The rice grains, freed from the hulls, are steamed, but the aggregation and gelatinization of the corns are avoided In order to prepare a malt, serviceable for

the brewer, from these grains, which are not capable of germination, and from which the ordinary diastatic action is consequently excluded, the mass of grains is mixed with the so-called "Tane kosi"—rice grains, which are permeated and covered with the mycelium and fruit spores of *Aspergillus Oryzæ*. In the moist and warm air there are developed on the rice at the end of about three days a white, velvety mycelium, which gives to the mass a pleasant smell resembling apples or pine-apples. Before the fructification of the mould takes place, a fresh quantity of steamed rice is introduced, and this also becomes covered with mycelium; this process is repeated several times. An aqueous extract of the koji-mass, thus generated, shows that a portion of the starch is converted into maltose and dextrin, and that some of the albuminous substances formerly insoluble in water are now soluble. Inasmuch as the above-mentioned *Aspergillus* is the only active factor, it has secreted different and diastatically active ferments; an aqueous extract of the mycelium of the mould actually shows diastatic properties. The koji-mass is mashed, twenty-one parts of koji being mixed with sixty-eight parts of rice, boiled by steam, and with seventy-two parts of water. This pasty mass is allowed to remain at about 20° C.; after some days it becomes clear, the saccharification of the starch and dextrin continually progresses, and at the same time a spontaneous and very violent fermentation takes place, being caused by a yeast-like ferment, which does not stand in genetic connection to the *Aspergillus*, and is not more closely known. At the end of two or three weeks the fermentation is finished, and the product is a clear, yellow, sherry-like liquid, containing 13 to 14 per cent. of alcohol.

5. The genus *Mucor* belongs to the most interesting of the groups of mould-fungi with which we have to deal, since it embraces species with very marked fermentative action. These generally occur as a grey or brown felt-like mass, frequently of very considerable height—sometimes several inches—in which can be distinguished, by the naked eye, fine yellow, brown, or black spherules.

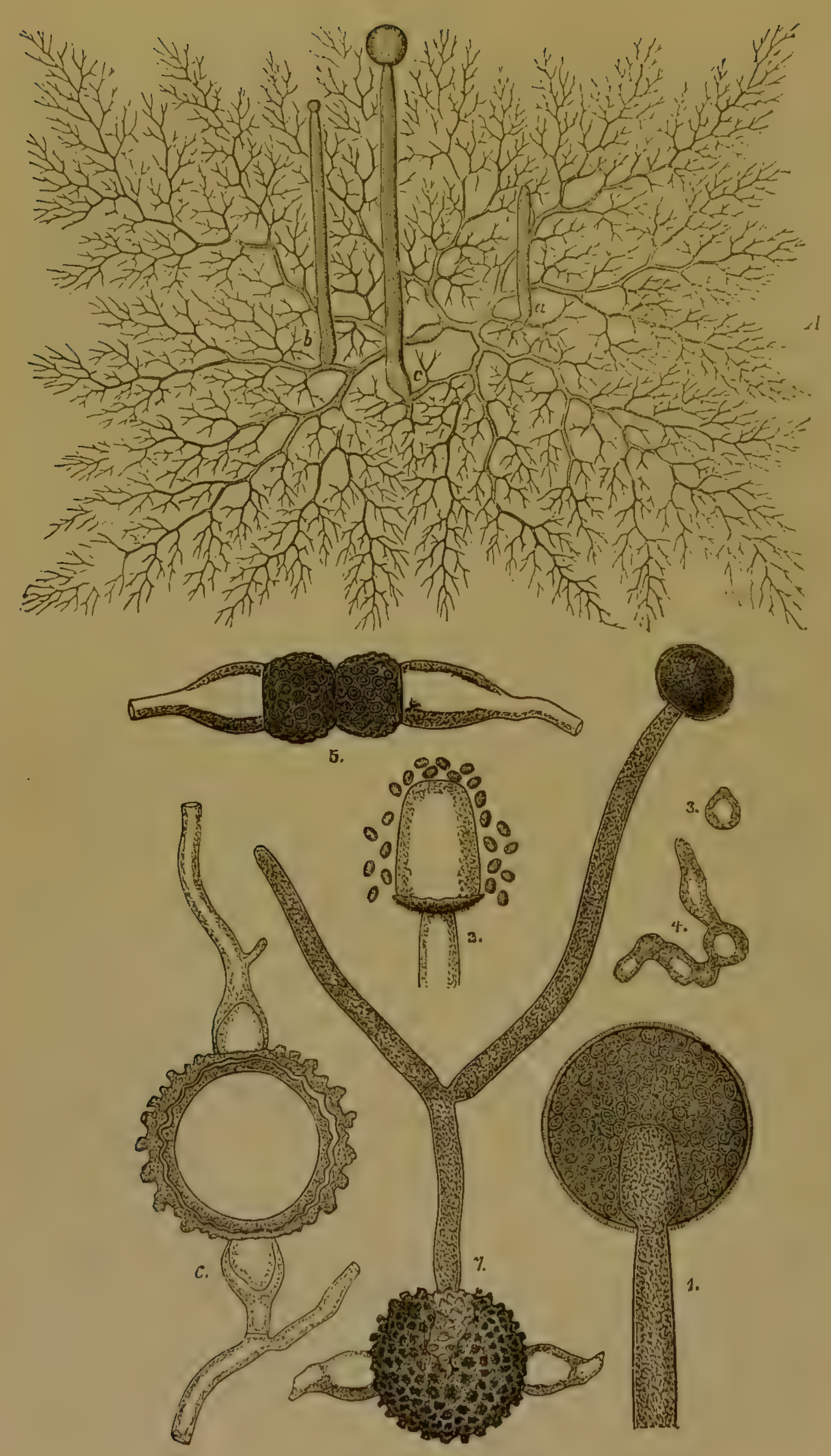


FIG. 17.—MUCOR MUCEDO. AFTER BREFELD AND KNY.

We give a description of the most frequently occurring species.

Mucor mucedo (Fig. 17), one of the most beautiful mould-fungi, and one which occurs very generally on the excreta of herbaceous animals, has a transparent white mycelium, which ramifies freely and with delicate filaments on and under the surface of its substratum, and which is, in certain stages of its development, without transverse septa and unicellular. From this mycelium there are thrown up simple vigorous branches, the sporangium-carriers; the points of these branches swell greatly, and below the swelling a transverse septum is finally formed, whereby the sporangium is defined from the sporangium-carrier. The transverse wall becomes arched upwards, and forms a short column—the columella—in the interior of the spherical swelling, whereby an inner space of peculiar form (1) results. The protoplasm of this space breaks up into a number of small portions, which surround themselves with a cell-wall and become spherical; these are the spores. At the same time these spores are clothed on their outer surface with small needle-shaped crystals of calcium oxalate. As soon as the ripe brown spores take up water the wall is dissolved, and the spores are scattered on all sides along with the swollen contents of the sporangium. The columella, which projected into the sporangium, still remains at the end of the stalk; this is now surrounded by a smaller collar (2), the remains of the outer wall of the sporangium. When the refractive spores fall on a favourable substratum, they swell very greatly and send out one or two thick elongations (3, 4), which quickly develop to a vigorous mycelium.

In addition to this method of reproduction, *Mucor mucedo*

Description of Fig. 17.

A, tree-like ramified mycelium with isolated thicker upright branches (*a*, *b*, *c*,). 1, Sporangium; 2, columella and spores; 3, 4, germinating spores; 5, 6, development of the zygospore; 7, germinating zygospore with sporangium.

and the other species possess another and a sexual method of reproduction, which takes place by means of a conjugation of two branches of the same mycelium. Two such short branches, growing adjacent to each other, form club-like swellings, and come in contact at their free ends (5). Each of the branches is then divided into two cells by a septum, and the end cells, which are in contact, coalesce by dissolving the original double wall which separated them. These two conjugated cells are either equal to each other in size, as with *Mucor mucedo*, or they are, as a rule, unequal to each other, as with *Mucor stolonifer*. The cells thus newly formed—zygospores (6)—quickly increase in size and swell to the shape of a ball (with *Mucor stolonifer* barrel-shaped), whereby the wall is thickened and stratified; externally it is coloured dark and covered with wart-like excrescences. These outer layers are very resistant to the attacks of acids. The zygospore is generally able to germinate only after a long period of rest; the germinating process bursts through the outer envelope, and finally develops the above-mentioned sporangium and spores (7). In the zygospore we thus find a resting-stage of the plant, an organ which by its structure enables the mould to preserve its life during periods which are unfavourable for its growth.

Mucor racemosus has a branched, many-celled sporangium-carrier, which can also attain to a considerable height. At the ends of the branches the greyish-yellow spores are developed. When this mould is cultivated in wort, the submerged mycelium swells irregularly, and there appear very many transverse septa, which define large barrel-shaped or irregular cells filled with highly refractive plasma. These cells—gemmæ—are easily isolated, and then take a spherical shape (compare Fig. 18; 7), as was first observed by Bail, and multiply by budding like the true yeast-fungi; the same takes place with the submerged spores (Mucor-yeast, spherical yeast). A similar formation of gemmæ may occur when the mould is cultivated on solid substrata.

Mucor erectus occurs, for example, on decaying potatoes, and microscopically has quite the same appearance

as *Mucor racemosus;* physiologically, however, it differs from this.

Mucor circinelloides (Fig. 18) has a very characteristic appearance. The mycelium (1) shows the remarkable branching which occurs with some of the varieties of *Mucor.* The main stem sends out short, root-like branches repeatedly forked and divided; at the base of these grow new mycelial branches, which become erect, and are able to form sporangia (2—5); the sporangium-carrier is branched. During its development considerable curves are formed, and to this the species owes its name of *circinelloides.* With this form, as with *Mucor spinosus*—whose chocolate-brown sporangia are distinguished by the fact that the columella is studded on its upper surface with pointed, thorn-like processes,—the mycelium, when submerged in a saccharine liquid, gives rise to a similar formation of gemmæ as *Mucor racemosus.*

Mucor stolonifer (*Rhizopus nigricans*) attains a very considerable size, and occurs very commonly; for instance, on succulent fruits. This mould is easily recognized, since the brownish-yellow mycelium sends aslant into the air thick vessels without septa; these attain a length of about 1 cm., then sink their points to the surface, and send out fine, greatly ramified vessels resembling rootlets into the substratum, whilst other vessels raise themselves perpendicularly, and develop sporangia; other branches again form new shoots. The black spore-case possesses a high dome-shaped columella, and develops a quantity of dark-brown round or angular spores. When these become free by the absorption of the case-wall, the columella is turned over on the hypha like an umbrella, of which the line of junction of the external wall remains in evidence in the form of a circular furrow.

The species of Mucor have, considered from our standpoint, a very considerable interest, since they are able to act, in different degrees, as true alcoholic ferments. As previously mentioned some of the species of Mucor immersed in a saccharine fermentable liquid alter their appearance very quickly; and whilst the mould thus approaches in its appearance to the

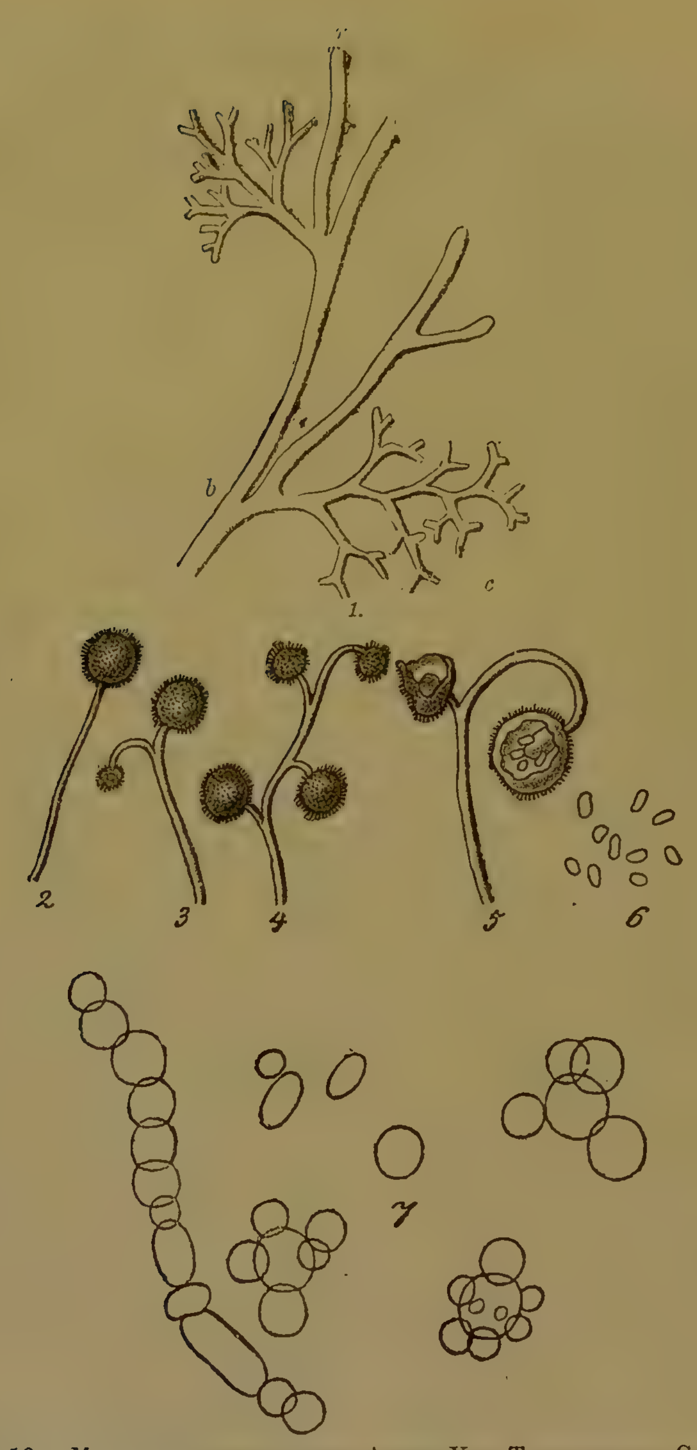


Fig. 18.—Mucor circinelloides. After Van Tieghem and Gayon.

1, Mycelium; *b*, main stem; *c*, root-like branches; *r*, axillary branches; 2—4, development of sporangium; 5, opened sporangium; 6, spores; 7, submerged mycelium and budding cells.

yeast-like fungi, it at the same time causes an actual alcoholic fermentation, since it produces alcohol and carbonic acid as the most important products of fermentation. If then the above-mentioned free members of the mould-fungus are brought to the surface of the liquid by the bubbles of carbonic acid, they are able to again develop the mould forms. The power of bringing about an alcoholic fermentation is possessed by almost all the species of Mucor, but in a different degree; still the fermentative power is not exclusively connected with the formation of the above-mentioned budding gemmæ, since these have not been observed with *Mucor mucedo* and *M. stolonifer*. The most active fermentative power is possessed by *Mucor erectus*, which forms in beer-wort 8 per cent. by volume of alcohol; it also excites alcoholic fermentation in maltose and dextrose solutions, but not in saccharose solutions, neither does it invert the latter sugar; this mould, however, sets up an alcoholic fermentation in solutions of dextrin, and converts starch into reducing sugar. *Mucor spinosus* in beer-wort gives as much as 5·5 per cent. by volume of alcohol; in maltose solutions marked fermentation phenomena are observed, and at the end of eight months the liquid contains 3·4 per cent. by volume of alcohol. Saccharose is neither inverted nor fermented; dextrose, however, is fermented, but in all cases this species possesses a feebler fermentative power than the preceding species. *Mucor mucedo* has only a comparatively feeble fermentative power both in wort and in maltose and dextrose solutions; saccharose is not fermented or inverted, but this species, like the former, gives a copious growth in such a liquid. *Mucor racemosus* ferments beer-wort, maltose—but not vigorously—and dextrose; this species secretes invertase and ferments saccharose after inversion.

Since *Mucor circinelloides* is without action on cane-sugar, whilst it exercises a very powerful fermentative action on invert-sugar, Gayon came to the conclusion that this mould might with advantage be employed to extract the cane-sugar from the molasses in the manufacture of sugar. So far, however, as I have been able to learn, this observation has not yet been practically employed.

6. *Monilia.*—Under this name there are found described in the mycological text-books a number of different fungi of comparatively simple structure; from a mycelium, which possesses a different colour according to the species, branches are thrown up, which give rise to a series of egg-shaped or elliptical spores. The genus has lately attracted interest, on account of one of its species, which Hansen has provisionally named *Monilia candida*, from Bonorden's description, showing very noteworthy physiological properties. It occurs in nature as a white film on fresh cow-dung, and on ripe succulent fruits. When added to wort it develops a copious growth of yeast-like cells, which have a resemblance to *Saccharomyces ellipsoideus* or *Sacch. cerevisiæ.* At the same time they excite a vigorous alcoholic fermentation, and form whilst this is still going on a mycoderma-like film on the liquid; the cells in this film extend more and more, and finally form a complete mycelium. In the first period the fungus formed only 1·1 per cent. by volume of alcohol, whilst *Sacch. cerevisiæ* gave 6 per cent.; but the *Monilia* continued the fermentation, and produced, at the end of six months, 5 per cent. of alcohol, whilst the yeast did not give more than the above-mentioned quantity.

Further experiments with this fungus led to the remarkable discovery that it does not possess the power of secreting the chemical soluble ferment invertase, and, therefore ferments saccharose *as* saccharose. As is known, saccharose has hitherto been considered to be not directly fermentable; Hansen has, therefore, proved that this statement possesses no general value; he has also shown that cane-sugar is directly assimilable. *Monilia* ferments maltose; in maltose and yeast-water it sets up an active fermentation; dissolved in pure water, however, maltose is not fermented; the ferment simply multiplies in this solution. Since *Monilia* does not secrete invertase, but is yet able to excite a fermentation in maltose solutions, it therefore follows that a previous conversion of maltose into dextrose is not in all cases necessary before the fermentation of the former.

The solutions containing the above-mentioned sugars show

during the fermentation the presence of carbonic acid and alcohol.

This ferment is distinguished by the ease with which it withstands high temperatures. In beer-wort and saccharose solutions it develops vigorously, and brings about an active fermentation at 40° C.

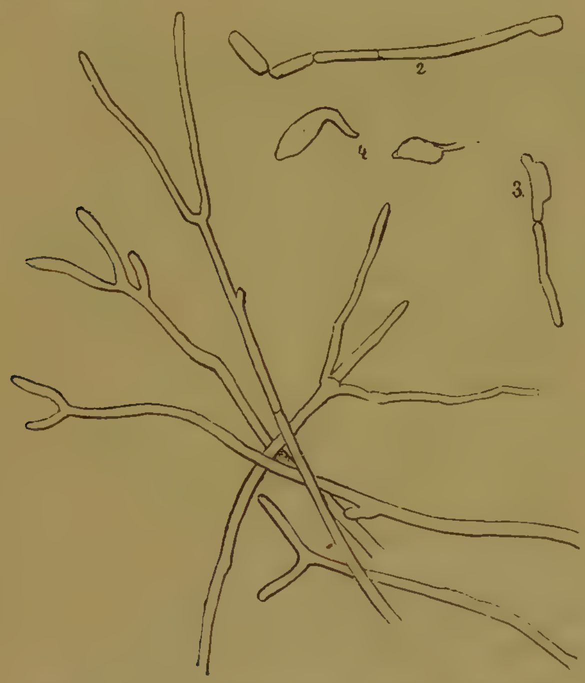


FIG. 19.—OÏDIUM LACTIS. AFTER HANSEN.

1, Hyphæ with forked partitions at the ends, and commencement of development of side branches; 2—4, conidia with germinating filaments; in 2 the germinating filament is already divided again into conidia, in 3 the conidium has given rise to a smaller and a larger germinating filament, the latter of which has separated itself at the point of formation; 4, two conidia, resembling yeast-cells in shape, beginning to germinate.

7. A mould-fungus, which has played an important part in the literature of the physiology of fermentation and in that of medicine, is *Oïdium lactis* (Fig. 19), the so-called lactic acid yeast.

Some authors have sought to establish the theory that this fungus is a stage in the development of species, which,

under other circumstances, occur in entirely other forms, and with quite different properties. It was thus brought in genetic connection with Bacteria, Chalara (see below), Saccharomyces, &c. &c. Both Brefeld and Hansen have carried out numerous researches with this fungus, and have undertaken culture experiments, which were continued for a long time, without producing any other than the ordinary Oïdium-form.

Fresenius correctly gave to this species the name *lactis* (milk), for universal experience goes to show that it has its ordinary place of abode in milk, where it can in the majority of cases be found. There has, however, up to now been no evidence brought forward that this mould-fungus stands in causal relation to the acid fermentation of milk. Further, it occurs spontaneously in various other liquids, and among these in the saccharine mixtures which find employment in the fermentation industries.

The often forked, branched, thin-walled, transparent hyphæ (1) form a thick white felt; in the uppermost portions of the filaments transverse septa arise close together, after which the single cells, filled with very refractive plasma, separate themselves as conidia (2). When the fungus grows on solid substrata, the hyphæ unite and form remarkable conical bodies. As a rule, the conidia in longitudinal section are rectangular with rounded corners; in a growth of this mould-fungus there are, however, nearly always found in addition spherical, round, pear-shaped, and quite irregular conidia (3, 4). These organs of multiplication, the only ones known, send out one or more germinating threads. The fungus may occur in beer, especially in samples poor in alcohol. Therefore, as the amount of alcohol increases, the conditions for its growth become more unfavourable; still, neither wort nor beer are exposed to the danger of being attacked to any extent by Oïdium, since it is not able to enter into the struggle with the concourse of organisms which at once arises when fermentable liquids are exposed to the germs of the air.

In numerous investigations with top-yeast, I found that this offers a very favourable nutritive material for this

fungus, especially when the yeast is standing in a quiescent state at the end of the fermentation. Sometimes a microscopic examination showed an enormously large number of the conidia. It is not known what influence such a growth exercises on the quality of the yeast and the beer; without doubt it is advisable to avoid the fungus as much as possible.

8. C. G. Matthews observed that the red colour which occurs on grains of malt, and more particularly when the quality is not very good, is produced by a *Fusarium*, most probably *graminearum*. He cultivated this mould on various substrata. The fascicular spores are spindle-shaped, curved, and uni- or multi-cellular; they are colourless or only very slightly tinted, but are embedded in the preparations in a strongly coloured mass. Matthews studied the germination of the spores, and states that this mould, cultivated in wort, produces in the submerged parts swollen and spherical cells resembling Mucor, which can bring about a fermentation. The formation of mould commences at the germinal end of the corn, and spreads from thence more or less over the surface. Generally, when such corns germinate they show an abnormal development, since they either send out only single rootlets with a sickly appearance, or the acrospire only. Whilst the spores of *Penicillium*, *Mucor*, *Aspergillus*, &c., are easily distributed over the malt on the floors by the air, the corns attacked by the *Fusarium* can, according to Matthews, only communicate the mould to the neighbouring corns, probably because the spores of this mould have a greater weight and more closely adhere to the original mould-growth than do the spores of the other organisms.

9. *Chalara mycoderma* (Fig. 20) is given in Pasteur's "Études sur la Bière" as one of the habitants on the surface of grapes. The mycelium forms a film on liquids, and consists of branched threads, which become greyish, and are often filled with highly refractive plasma, which develops at different points conidia of unequal form and size. Cienkowski, in his memoir on the moulds, first thoroughly

described the film growth of *Chalara.* Hansen found that this fungus does not thrive in ordinary wort and lager-beer; if these liquids are, however, diluted, the mould develops freely, and forms a thick, brilliant, viscous and tough membrane.

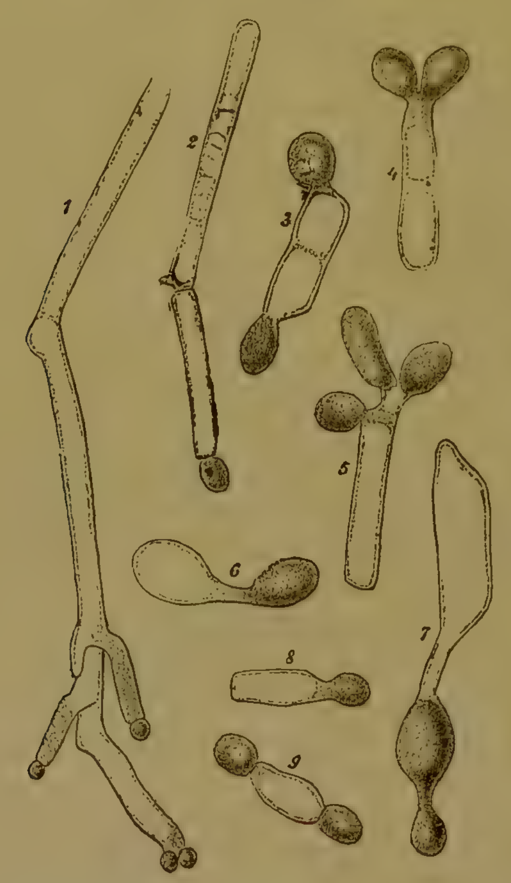


FIG. 20.—CHALARA MYCODERMA. AFTER HANSEN.

1, A branched hypha, the end-limb of which is throwing off conidia; 2, a hypha, at the upper cell of which a sterigma, which has separated conidia; 3—9, various forms of members of hyphæ, which are separating conidia.

10. A mould-fungus concerning which a great deal has been written in the literature of our subject, but whose practical importance, however, certainly stands in inverse ratio to the attention bestowed on it, is *Dematium pullulans*

(Fig. 21), which was first described by de Bary, and later more minutely by Loew. It frequently occurs on fruits,

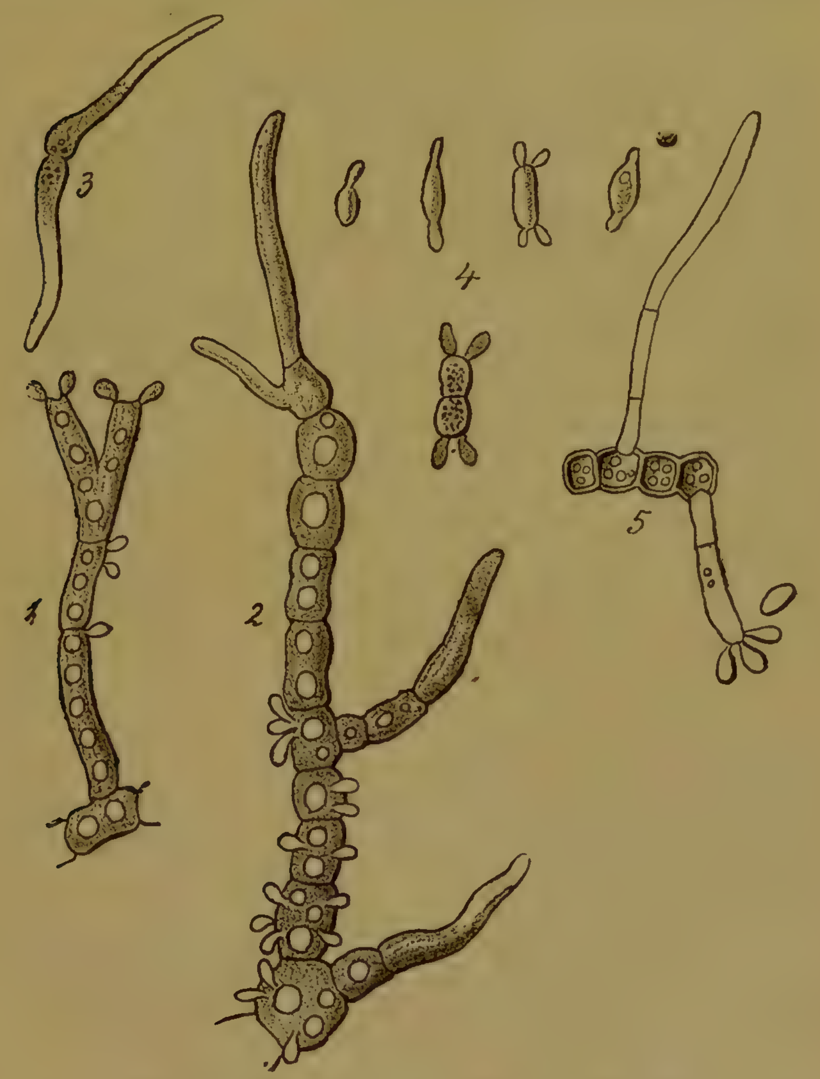


FIG. 21.—DEMATIUM PULLULANS. AFTER LOEW.

1, 2, Growing mycelial threads with yeast-like cells; 3, cells of the latter kind developing to mycelial threads; 4, cells with yeast-like budding processes; 5, appearance of yeast-like cells on the germinating threads of the brown-walled cells.

especially grapes, and has a branched mycelium, on which buds are thrown out; these have a striking resemblance to

ordinary yeast-cells (4), and are able either to propagate through many generations by yeast-like budding, or to form germinating threads, which give rise to a mycelium (3). When this has attained a certain age, it then forms numerous thick, closely-situated, transverse septa, and gradually becomes brown or olive-green (5); in this we have the resting stage of the plant. In Hansen's air analyses *Dematium* was very frequently found in the wort to which the air had access, from spring until late autumn; he observed that when the mould was sown in a saccharine liquid, it at first only developed mycelial threads; after some time, however, the yeast-like cells were separated, without giving rise to an alcoholic fermentation. Pasteur has very fully treated of this plant in his "Études sur la Bière." Since it occurs in such copious amount on the surface of grapes, where the wine-yeast is found, and since this often has exactly the same appearance as the yeast-like cells thrown off by *Dematium*, it might be supposed that the conidia and the cells of wine-yeast (*Saccharomyces*) were identical. Pasteur speaks variously in different places in the above-mentioned work on this point, since he in certain relations only puts forward this connection as a supposition; in other places, however, he considers it as something self-evident. We have here again an example of the endeavours mentioned formerly to connect the yeast-fungi (*Saccharomycetes*) with the mould-fungi. According to the present methods of research, the question allows of no more doubt. The true wine-yeast can, under certain conditions, to be described later on, give rise to spores in their interior. Under the same conditions the conidia of *Dematium* develop no spores, and are therefore distinguished from the wine-yeast.

11. Finally, we have to mention a mould which may occur in fermentable liquids and in fermenting vessels—*Cladosporium herbarum*. The mould sometimes occurs in very large quantities in fermenting vessels. I found, some years ago, in a bottom-fermentation vessel, the cover and a portion of the sides thickly covered with small black patches; these consisted of this mould, whose conidia I therefore always

found in the yeast. The plant consists of a yellowish-brown mycelium with short, straight, stiff, and brittle threads, of which those growing erect are able in their extreme portions to produce conidia of very varying form—spherical, oval, cylindrical, straight, or curved. The systematic position of the mould, and its possible genetic connection with other known moulds, is just as little established as its action on nutritive liquids. Eriksson states that rye is sometimes attacked by *Cladosporium*, and that the mould, consumed in such bread, or in beer, may give rise to diseases in the human organism.

Concerning these or at least closely related forms, Zopf gave some morphological investigations with numerous illustrations in his memoir on Fumago. This last-mentioned "black-dewlike" mould occurs very frequently on parts of plants. Frank correctly says: We are still quite in the dark with regard to specific differences, the reason of which is especially to be found in the frequent polymorphism of the organisms, and the circumstance that the individual evolution-forms are almost never found together.

CHAPTER V.

THE SACCHAROMYCETES, OR YEAST-FUNGI.

Introduction.

It does not lie within the scope of a book of this description to give a detailed historical review of the knowledge of bygone times. Only so much should be given as is sufficient for a proper understanding of the present position of the subject under consideration. As the work of the last decade has really proceeded more or less directly from questions arising in practice, the results obtained are, therefore, called forth with the full assurance that they will be transferred to industrial operations, and thereby greatly benefit technology. This can naturally, however, only take place when the value of the scientific results are correctly understood. The object of the following *résumé* is to facilitate such an understanding.

The term "alcoholic ferment," employed in a general sense, is very comprehensive. Both mould-fungi, bacteria and budding-fungi, are able to bring about an alcoholic fermentation. In the following we are only concerned with the last-mentioned. Among these there may occur some which also form mycelia, whilst with others this form of development is unknown; from among these last-mentioned budding-fungi without mycelia a group is again separated, on account of the power of the members to form endogenous spores, under the name of *Saccharomycetes*. Quite recently, however, the formation of mycelia has been detected in some few Saccharomycetes.

In the year 1870, Reess published his "Botanischen Untersuchungen über die Alcoholgärungspilze." In this work it was shown for the first time, what is intimated above, that

there is found among the budding-fungi a group of organisms which are characterized by possessing the power of forming endogenous spores and not developing a mycelium, whilst their alcoholic fermentative activity is of comparatively subordinate importance. We thus get a sharp and distinct separation between the Saccharomycetes and all the remaining budding-fungi. Reess further constructed a system of the Saccharomycetes, which he based on the size and form of the cells alone; this classification of species, based entirely upon pure microscopical appearances, unfortunately proved to be faulty; we therefore do not learn from this work how to distinguish the species one from another. The work of Reess has consequently not been of any real practical importance—it was even disputed for many years whether spores occurred in yeast used for practical purposes or not; finally it was put forward as a dogma—by Brefeld, for instance—that cultivated yeast, which was continually compelled to multiply by the formation of buds, had lost this power. Hansen's investigations first brought order into this confusion.

Pasteur's "Études sur la Bière" appeared in the year 1876. Our knowledge of the phenomena connected with fermentation was considerably advanced in many directions by this work. It was here clearly and incontestably shown what power the microscopic organisms possess, and it was strongly emphasized that bacteria especially can exercise a decided influence on the course of fermentation and on the character of the resulting liquid. The budding-fungi were also treated in detail, and it was suggested, as had earlier been done by Bail and other investigators, that single ill-defined members of this group can influence the nature of the product of fermentation in different ways. What Pasteur stated on this point are merely suggestions in two entirely opposite directions. This especially appears in his observations on the so-called caseous yeast and aërobian yeast. Possibly we have here the account of distinct, peculiar species of yeast; possibly, however, only of forms produced by a certain treatment of ordinary brewers' yeast. It must not be forgotten, however, that he defines his standpoint with perfect clearness, and even points out

in what direction the reason will be found why the question could not be decided, namely, that at that time it was not possible to determine whether the yeast with which he worked consisted of one or more species; the methods for the pure cultivation of yeast were, in short, at that time not yet discovered. A true orientation in this world of micro-organisms is consequently not found in this learned work; it is not possible in any part of Pasteur's statements to find such characteristics for the budding-fungi that a scheme of analysis could be based upon them. Pasteur considers all the budding-fungi with any distinct power of alcoholic fermentation as Saccharomycetes; it is nowhere clear whether the description concerns the true Saccharomycetes or the other budding-fungi. These yeast-fungi, which in our present system may belong to very different divisions, were formerly considered to be stages of development of mould-fungi resembling Dematium, without any evidence for this belief being given. Whether there are different species of these budding-fungi (Saccharomycetes, Torula, Dematium, &c.) or not, Pasteur leaves undetermined. His treatment of the botanical problems mentioned above must, on the whole, be considered as breaking down on these important points.

As, however, Pasteur's work always retains its importance for technical purposes, on account of the force with which the influence of bacteria on the fermentation industries is made manifest, so it also possesses a great theoretical interest, especially from the new theory of fermentation enunciated therein, which at the time rightly attracted great attention.

Contrary to Brefeld, who maintained that yeast could not multiply without free oxygen, and Traube, who correctly allowed that yeast was able to develop without free oxygen, but asserted that it then required soluble albuminoids in the liquid for its cell-formation, Pasteur enunciated the theory that the fermentation organisms formed a group of plants whose functions as ferments are directly "a necessary consequence of life without air, of life without free oxygen," and further, that such a fermentation can take place in pure sugar solutions. He maintained that the reason why Brefeld

could not get yeast to develop in a moist chamber in an atmosphere of carbonic acid was because he worked with old yeast-cells, whilst the multiplication of yeast without free oxygen is only possible when the cells are very young. The smallest amount of free oxygen which is present in the liquid to which the yeast is added "rejuvenates the cells and makes it possible for them to again exercise the power of budding, to preserve life, and to continue their multiplication without the presence of air."

Pasteur therefore makes a definite separation between the two classes of organisms: *aërobian*, those which cannot live without the presence of free air; and *anaërobian*, those which may be completely deprived of air for a long time. These latter are "ferments in the true sense of the word."*

It was incorrectly considered that the presence of alcohol and carbonic acid among the products of a fermentation must absolutely presuppose the influence of "the organisms of alcoholic fermentation in a true sense." It was shown, however, in the experiments first made by Lechartier and Bellamy, and later confirmed and extended by Pasteur, that when grapes, oranges, and other fruits, on which no yeast-cells are present, are preserved in a hermetically closed space filled with carbonic acid, a development of alcohol and carbonic acid takes place. "The fermentative capacity is, therefore, not a condition of existence with yeast; the fermentative power is not peculiar to cells of special nature, is no permanent structural characteristic, but is a property which depends on external conditions and on the mode of nutrition of the organism."

"In short, fermentation is a very general phenomenon. It is life without air, or life without free oxygen, or, more generally still, it is the result of a chemical process accomplished on a fermentable substance—*i.e.*, the substance capable of producing heat by its decomposition, in which process the entire heat used up is derived from a part of the heat that the decomposition of the fermentable substance sets free. The class of fermentations properly so-called is,

* On this recently very debated question, compare page 43.

however, restricted by the small number of substances capable of decomposition with the production of heat, and at the same time capable of serving for the nourishment of lower forms of life when these are deprived of the presence and influence of air." This is in brief the celebrated theory of fermentation enunciated by Pasteur.

The numerous experiments carried out by Pasteur and his disciples on the influence of aëration, experiments which are much quoted in the zymo-technical literature, are connected with this.

Of Nägeli's manifold work on the lower organisms we will only mention, as connected with the foregoing, the molecular-physical theory of fermentation put forward by him, and which is really a modification of that of Liebig. Whilst Pasteur explains fermentation as the result of an activity taking place in the cell, Nägeli defines fermentation as a transference of the conditions of vibration of the molecules, atomic-groups, and atoms of different compounds (which themselves undergo no change) of the living plasma to the fermentative substance, whereby the equilibrium in these molecules is destroyed and they are consequently decomposed. In fermentation, therefore, the vibrations of the plasma-molecules are transferred to the fermentative material. The causes of fermentation are found in the living plasma in the interior of the cell, but they operate at a moderate distance outside the cell. The decomposition of sugar—especially into alcohol and carbonic acid—takes place to a small extent within the yeast-cells, to a greater extent outside them. This theory thus stands in direct opposition to that of Pasteur.

In Brefeld's numerous mycological communications the budding-fungi occupy a fairly prominent place; thus this naturalist showed that many Ustilagineæ (smuts) could assume a budding-fungi stage. It was, however, not proved whether these forms have the endogenous spore-formation characteristic of the Saccharomycetes, nor whether they possess a marked fermentative activity.

In the whole of the different theories of fermentation, the central point of none of the following questions is touched

upon: How comes it that in these microscopic cells the plasma, which has the same appearance in the different species, yet sets up in the one cell an acetic acid fermentation, in the other a butyric acid fermentation; in this cell is able to directly ferment cane-sugar, in that, however, only after a previous inversion? The causes of these different activities of apparently the same plasma are still an unsolved problem.

Consequently our knowledge of the alcoholic yeast-fungi was very deficient and unreliable at the time Hansen commenced his investigations; these investigations had to be undertaken experimentally from the very foundation. Hansen has done this in the work which he has carried on for nearly ten years, and the greatest interest is attached to the question: By what methods did he arrive at that knowledge of the Saccharomycetes, which even at present—and not only from a scientific standpoint—has given us a deeper insight into the varied phenomena of fermentation, and which has carried us so far that a reform of the yeast question in the industry has, in consequence, been carried out? This question we will, therefore, now discuss.

HANSEN'S INVESTIGATIONS.

When Hansen published in the year 1878 his paper on "Organismen in Bier und Bierwürze," he pointed out the uncertainty which prevailed in the works of earlier authors as to the true Saccharomycetes; he emphasized the fact that it was not possible to proceed further along the path pursued by former investigators; that most of the investigations commenced by Pasteur and Reess must, if they would be carried farther, be attacked from an entirely different point of view. It was only in the latter half of the year 1881 that he was able to find a key to this problem. The problem was to devise a method by which one could obtain growths, each proceeding from *one single*

cell, in order in this way to prove by experimental investigations whether the pure culture thus obtained possessed constant characteristics, that is to say, in how far the Saccharomycetes occurred as species, varieties, or races; and in case this was confirmed, to determine what these constant characteristics were. When this problem was solved, it would then be possible for the first time to devise a solution of the practical question of a method for the analysis of yeast.

As this purely scientific work was called forth by technical requirements, the results of the work at these problems must again return to influence technical practice.

1. Preparation of the Pure Culture.

We have already shown, in the earlier pages of this work, that the idea was expressed on various sides, that the condition for any exact knowledge of the microscopic organisms, of which we find hundreds or thousands in every drop when examined under the microscope, consists entirely in the separation of the individual cells, and working with the pure growth proceeding from one of these. At the same time we have briefly intimated the different methods which have been proposed for this purpose.

Hansen has repeatedly shown in his papers that, in all cases, the only certain method is that which starts from the individual cell, and secures the beginning from this cell. At first, the dilution methods used by Nägeli, Fitz, and others (compare page 20), were, with some modifications, employed for this special purpose. The method proposed by Hansen is shortly as follows:—A vigorous alcoholic fermentation is started in a Pasteur's flask; the growth of yeast is then diluted with sterilized water in the desired proportion, and the number of cells in a small drop of the thoroughly shaken liquid is counted. The counting is, in this case, quite easily carried out by withdrawing a drop from the liquid and transferring it to an ordinary microscopic cover-glass, in the centre of which some small squares are

engraved, and which serve as resting points for the eye; the drop must not be allowed to flow beyond the limits of the squares: the number of cells occurring in the small drop are then counted. There are found, for instance, ten cells; a similar sized drop of the liquid, which has been again thoroughly shaken, is then transferred to a flask containing a certain known volume (20 c.c. for instance) of sterilized water. It is thus probable that this flask contains about ten cells. The contents are now subjected to a prolonged and vigorous shaking, and 1 c.c. of the liquid is quickly transferred to each of twenty flasks containing nutritive liquid; ten of the twenty flasks have now probably each received *one cell*. The whole is, however, to this point only a matter of probability. If the flasks are now allowed to remain for the further development of the cells, there is then a prospect that a pure culture may be obtained in at least some of the twenty. This, however, cannot be depended upon with any certainty. To Hansen belongs the credit of having supplied the links which first gave certainty to this experiment. Thus, if the flasks, inoculated as above, are very vigorously shaken, and then allowed to remain perfectly still, the single cells sink to the bottom or attach themselves to the walls of the flask. It is self-evident that if the flask contains, for example, three cells, these three cells are, or at least in the majority of cases, separated from each other by the vigorous shaking, and consequently each takes up a separate position on the bottom. Then, at the end of some days, the flasks are carefully lifted and examined, and it is noted whether one or more white specks have been formed on the walls of the glass. If only one such speck is found, we have then a pure culture. It is self-evident that by means of this method we are also in a position to directly inoculate a single cell into any desired flask with nutritive solution.

By the aid of an extended knowledge of the species, it was possible to submit this method to a searching test, and it was found that Hansen's assumption was correct.

If it is desired to isolate from a mixed growth of different species those which are in an enfeebled condition, it is then

necessary, as Hansen has pointed out, to employ this method, since we always work with liquids, *i.e.*, with the most favourable medium of nutriment for the desired organism.

If, however, the opposite is the case, and we wish to separate from a mixed growth the species which is present in a state of vigorous development, and whose growth is consequently not dependent on especially favourable conditions of nutriment, we can attain our object more quickly, and with less expense, by the employment of a solid nutritive substratum—in this case gelatine and wort. It has been proved that the addition of gelatine to a liquid decreases its value as nutritive material for the bacteria and yeast-fungi; this disadvantage is, however, of less importance in the particular case with the above-mentioned conditions, namely, that the growth from which it is desired to make a pure culture is young and vigorous. An advantage of this method, as it is employed by Hansen for the study of the alcoholic ferments, is that it is possible to directly observe the individual under the microscope and follow its further development, since the gelatine plate is enclosed in a moist chamber. If we refer to the earlier part of the book (chapter I.) we shall find that this method is described in connection with the methods employed by other observers.

2. The Analysis of Yeast.

Throughout the entire series of Hansen's work one fixed idea obtains, namely, that the shape, the relative size, and the appearance of the cells, taken by themselves, are not sufficient to establish the characters of a species, since the same species, under different external conditions, can occur in an entirely different manner, and with an entirely different appearance,* but the form and phases of development,

* Hansen mentions as a very marked example of this kind, that the cells of a *Sacch. cerevisiæ* (bottom yeast), after long, slow development, maintain the ordinary well-known appearance when they are propagated in wort at about 27° C., whilst when cultivated at 7·5° C. they give closely developed colonies with mycelium-like branchings.

considered from another side, give very important points of difference for the different species. Thus it is found that there are limits to the influence which can be exerted on the cells, and that the different species behave in a different manner under the same treatment; this can only be explained by assuming that hereby the intrinsic, indwelling characters of the special cells themselves exert an influence.

In the following we give a short account of the different ways in which the characteristics of the species are determined by Hansen.

a. The Microscopic Appearance of the Sedimentary Yeast.

The first examination of a growth of yeast is, of course, always made by bringing a sample of the yeast under the microscope for preliminary information. As an example of what can be ascertained in this way, we may instance the following figures (Figs. 23, 26, &c.) which exhibit the sedimentary forms* of the six species of Saccharomycetes which have been specially treated by Hansen.

* In addition to the ordinary dough-like sediment, Hansen also sometimes found a membrane-like wrinkled layer on the bottom of the inoculated flasks. Thus *Sacch. Past.* II. develops a membranous sediment at temperatures below 7° C., *Sacch. Past.* III. below 22° C., and in a very marked manner between 14° and 1° C. especially. This membranous sediment consists chiefly of strongly developed mycelium-like colonies, and has altogether a different appearance to the ordinary culture.

When cultures of Saccharomycetes, which had been grown several months in saccharose (cane-sugar) solutions, were transferred to wort, the same observer often noticed a similar sediment when the cultures were carried on at 25-28° C. for the three *Pastorianus* species, never, however, under these conditions with the three other species. At the same time, with the membranous sediment there occurred cheesy lumps; even a sediment formed exclusively of these was frequent. In both cases this sediment was much less dense, and by vigorous shaking the liquid became brilliantly transparent, even during the most vigorous fermentation. By continued fresh fermentations with wort the phenomenon disappeared, and we have here again an example of a provisional physiological change, since it is possible to produce by a certain treatment, growths of different species of caseous yeast (Pasteur's *lévure caséeuse*).

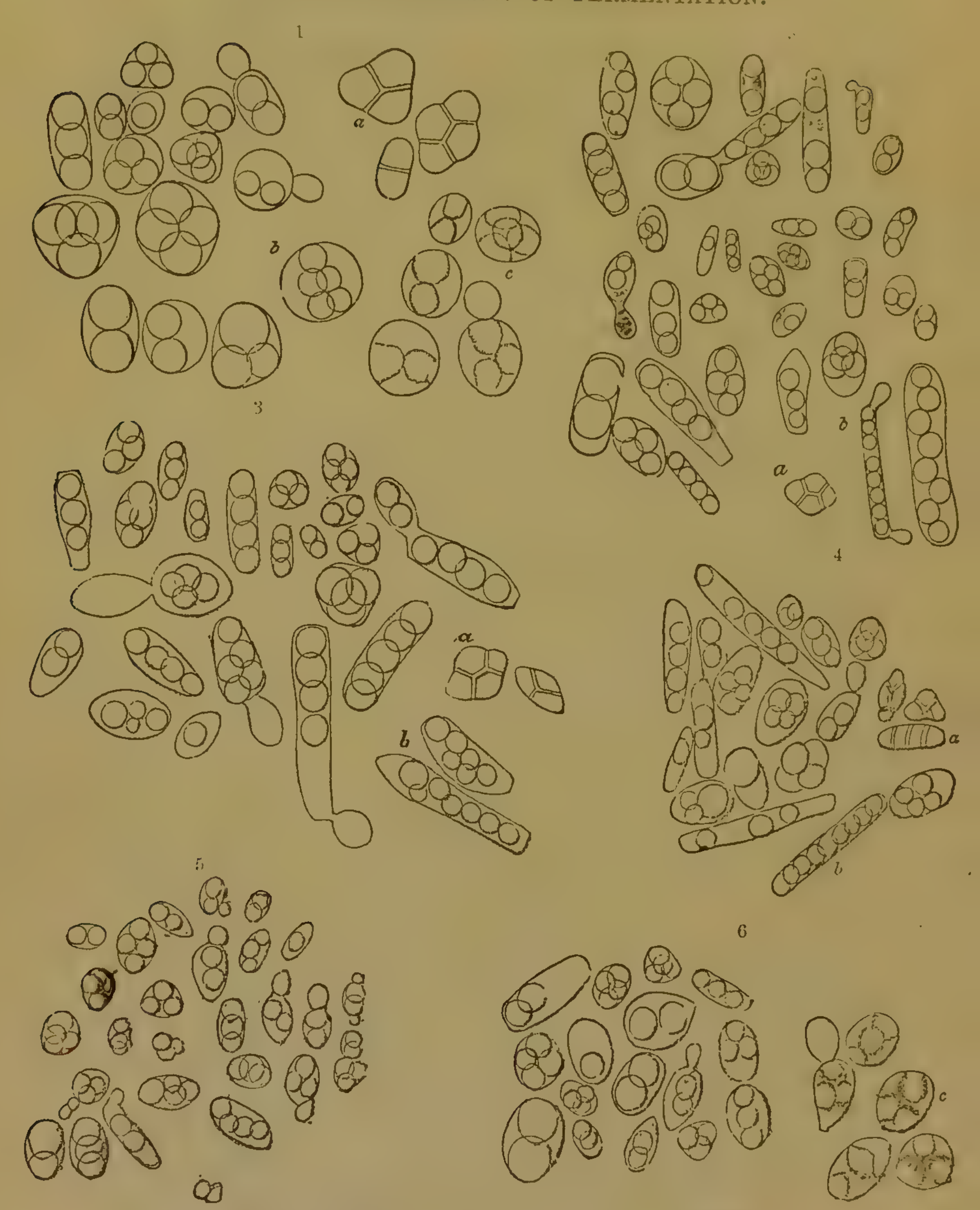


FIG. 22.—SACCHAROMYCETES WITH ASCOSPORES.

1, Sacch. cerevisiæ I. ; 2, Sacch. Pastorianus I. ; 3, Sacch. Past. II. ; 4 ,Sacch. Past. III. ; 5, Sacch. ellipsoideus I. ; 6, Sacch. ellips. II. ; *a*, cells with partition-wall formation ; *b*, cells with a larger number of spores than usual ; *c*, cells with distinct rudiments of spores. (After Hansen.)

The growths in these cases were obtained by transferring the cells, which were cultivated for some time in wort, to fresh wort, and causing a vigorous growth to be set up in twenty-four hours at 26° to 27° C. If we now compare, for instance, the figures of *Saccharomyces cerevisiæ* I. with the three *Pastorianus* species, we find a very considerable difference in the forms. The *Sacch. cerevisiæ* consists chiefly of large round or oval cells. The *Pastorianus* species form mostly oblong sausage-shaped cells. It is, however, a very different matter when the cells of the first species are mixed with cells of one of the other species ; it is then, judging by the form only, not possible to distinguish the larger and smaller oval and round cells of the *Pastorianus* species from many of the *Sacch. cerevisiæ* cells. The two species of *Sacch. ellipsoideus* I. and II. possess (Figs. 32 and 34) a majority of oval and round cells; sausage-shaped cells are, however, present, and consequently it is in this case also impossible to determine the species by the form when they are mixed with *Sacch. cerevisiæ* or *Sacch. Pastorianus.* No great points of difference were found by the direct measurements of these sedimentary forms. The greatest diameter of *Sacch. cerevisiæ* I. was 11—5, mostly 8—6 micromillimetres ;* the three *Pastorianus* species had a greatest diameter of 17—2½ μ, most frequently 8—7 μ ; the *Sacch. ellipsoideus* I., 13—2½, frequently 7—6 μ; the *Sacch. ellips.* II., 11—3, most frequently 8—7 μ. On the other hand, an examination of the six groups of illustrations of pure cultures at once shows that we have here three different divisions of budding-fungi, of which one is represented by *Sacch. cerevisiæ*, whilst the second embraces the three *Pastorianus* species, and the third the two *ellipsoideus* species. This much, but only this much, is possible by a purely microscopic examination, and, as we must again point out, only under the conditions of cultivation given above.

* The micromillimetre is denoted by the symbol μ, and its value is 0·001 mm.

b. Formation of Ascospores.

The first step towards a true analytical method in the field of the Saccharomycetes was made when Hansen commenced his investigation of the endogenous spore-formation among the true Saccharomycetes. We give a short *résumé* of the experimental method adopted by Hansen, and the results obtained in general.

The formation of spores in yeast-cells has been investigated by various naturalists—de Seynes, Reess, Engel, Schumacher, Münz, Brefeld, van Tieghem, Wiesner. In addition to the description and mode of preparation of the spores, we often find among the above-mentioned authors some curious theories; in all essential particulars, however, these statements are not found to stand the test of renewed examination. We have already remarked that Reess endeavoured to establish a classification of the Saccharomycetes on the form and size of the cells and spores. He further stated that such a formation of spores generally took place if the yeast remained a long time in the brewery without being attacked by mould-fungi; also, that the development of spores was favoured by a low temperature, and that the "top" yeast could only form spores after conversion into "bottom" yeast. Van Tieghem considered he had established by his researches that spores were disease formations, which occurred when the cells were attacked by bacteria—which was, like the statements of Reess just mentioned, not correct.

Wiesner considered that the development of ascospores might be practically used as a means for the detection of the adulteration of pressed barm. The cells of the latter yeast were not able, according to him, to form spores, whilst, on the other hand, the beer yeast used for adulteration possessed this power. Therefore, if on culture the pressed yeast were found to yield ascospores, it must be considered adulterated. As a matter of fact, however, both pressed yeast and beer yeast possess the power of forming spores. Finally, Brefeld stated that only "wild yeast" was able to develop spores, and that the "cultivated yeast" had lost this property on

account of the special conditions of industrial culture. This also is, as before remarked, at variance with the facts actually observed.

The sole result which the above-mentioned workers and all others who touched the subject obtained was simply the fact that under certain, but still unknown, conditions the cells of Saccharomyces can form spores in their interior.

In order to obtain this formation, Hansen cultivated the yeast in the same manner as given above. A small quantity of this yeast was transferred to a previously sterilized gypsum block; this block was placed in a flat covered glass, and was then maintained moist by filling the glass half full of water.* If now it is simply desired to bring about the formation generally, the apparatus can be allowed to remain at the ordinary room temperature.

The spores then, after the lapse of some time, occur as spherical or oval bodies in the cells (Fig. 22). Such cultures often show the so-called "partition-wall" formation also. This proceeds from a swelling of the spores, whilst the membrane of the mother-cell is strained so tightly and strongly round the spores that it is only by means of chemicals, or by the bursting of the cell, that it is possible to clearly detect the wall of the latter; at the same time, the swollen spores exercise a pressure against each other, and their walls come into very close connection at the surfaces of contact; sometimes, also, the remainder of the plasma of the mother-cell will be pressed like wedges or walls between the spores; then either the spore-walls alone or the just mentioned plasma-walls, or both together, form the "partition-wall." Ordinarily the spores can be set free by means of pressure on the cover-glass; there are, however, cases where this is not possible, since the cells are bound together so fast that they are crushed by a violent pressure, and in this case a separation between the walls is not noticeable, even when high powers are used; we therefore have here an

* The ascospores may also be obtained when the yeast is spread on sterilized solid gelatine kept in a damp place; also in yeast-water and sterilized water.

actual growth by which yeast-cells have been converted into multicellular bodies.

The germination of the spores takes place in such a way that they swell very considerably, and develop buds like the vegetative cells.

Hansen's investigations were carried out in order to ascertain the laws and conditions for this development in the cells, and then to obtain information as to what influence different temperatures exercised on the formation of spores, and also to determine whether the different species behaved alike or whether it was possible to obtain different characteristics. It was, therefore, necessary to determine the following points:—1. The limits of temperature, *i.e.*, the highest and lowest temperature, within which spores could be formed. 2. The maximum temperature, *i.e.*, the temperature at which spores appeared the most quickly; and, finally, 3. The relation of the temperatures lying between these points.

In determining the required degree of temperature, the observation is taken at the moment when the cells have distinctly commenced to form spores. The spores then show themselves (compare Fig. 22, 1 and 6) as bodies with irregular contour, formed from the protoplasmic part of the cell contents. It is not possible to employ the appearance of ripe spores as the moment of observation and determination, since there exists no criterion for complete ripeness.

Hansen's results established the following necessary conditions:—

1. Plentiful supply of air and a moist surface.
2. The employment of only young, vigorous cells; cells other than these do not show the formation.
3. The maximum temperature for the six specially-examined species lies in the neighbourhood of 25° C.

The formation of spores takes place slowly at low temperatures, more quickly, however, as the temperature rises until a certain point is reached; when this point is passed, the development again decreases, until it finally stops altogether.

The lowest limit of temperature for the six species at first investigated was found to lie between 0·5 and 3° C., the highest at 37·5° C. It was found, for these six species, the intermediate temperature and time relations of which were determined by Hansen, that when these two values were graphically represented, the degrees of temperature being taken as abscissæ and the intervals of time as ordinates, curves were obtained, which had essentially the same form for all six species. They sink from the ordinates of the lowest temperatures to the axis of the abscissæ, and then rise from these; at the same time, however, it results from these curves that the cardinal points determined from the highest and lowest temperatures give characteristic distinctions for the species, that is to say, the limits of temperature, within which the formation of spores in the single species can take place, are different (compare the following chapter).

With regard to the time which is required for the first indications of the spore-formation in the six species investigated under the same conditions of temperature, the following was observed. At the highest temperature the development in all the species required about thirty hours; at 25° C. also there was no important difference in time; but as the temperature was lowered very evident differences occurred. Thus *Sacch. cerevisiæ* I. does not develop spores at 11·5° C. until after ten days, whilst *Sacch. Pastorianus* II., under the same conditions, shows the formation after seventy-seven hours, and so on.

In all determinations of this nature the condition of the cells exercised a very great influence, according to whether they had been grown at high or low temperatures, were old or young, feeble or vigorous, &c., &c. From this it follows that the composition of the nutritive liquid also exercises an influence. It must especially be emphasized that the formation of endogenous spores only takes place when the cells have free access to the air. Therefore it is a necessary condition for methodical comparative investigations of this nature that the cells should always be previously cultivated in the same manner. If these external conditions are varied,

the limits for the reactions of the species must at the same time be correspondingly determined.

After Hansen had made these observations, the method was at once employed for the analysis of brewery yeast. The pure cultivated yeast (No. I.) used in the brewery of Old Carlsberg forms, at 25° C., spores much later than all the Saccharomycetes capable of producing diseases in beer which have hitherto been examined. Consequently there was, for the first time, a means of carrying out an actual analysis in this direction.

Holm and Poulsen, by means of investigations performed for the purpose of determining how far Hansen's analytical method held good for analyses conducted for technical ends, arrived at the conclusion that it was possible in this manner to detect with certainty a very small admixture of wild yeast, about 1-200th of the entire mass (Carlsberg bottom-yeast No. I.). It was shown in earlier investigations made by Hansen that when, for instance, the two species, *Sacch. Pastorianus* III. and *Sacch. ellipsoideus* II., which are able to bring about yeast-turbidity in beer, are only present in a proportion of 1-41st of the pitching yeast, the disease is not developed—that fermentation and storage take place in a normal manner ; consequently an analysis of yeast by means of the ascospore formation, according to Hansen's method, is more than sufficient to show the presence of these ferments.

This method also possesses the advantage that the analysis can be performed with mixtures, and in a short time. Thus, in the case mentioned above, a distinct answer can always be obtained at the end of forty hours by a culture at about 25° C.

As mentioned above, the cultivated varieties or races behave differently, and accordingly the exact arrangement of the experiment has to be determined in each case. The bottom-fermentation yeast No. I. (Old Carlsberg), cultivated in a pure state by Hansen, is, as before stated, best examined at about 25°C. A gypsum block cultivation of some of the other races carried out at the same temperature will, however, only give with great difficulty any indication of an admixture of wild yeast, because at this temperature these races develop

spores only a very short time after the wild yeast. If, however, such a culture is maintained at about 15° C., it is seen that the pure cultivated yeast develops spores much later than the wild-yeast cells.

According to the investigations carried out by Holm and Poulsen, the cultivated races of yeast used in bottom-fermentation breweries can be grouped under one of the two above-mentioned types, since they must be analysed either at 15° or at 25° C.

c. The Formation of Films.

By the observation of the formation of films among the Saccharomycetes, Hansen has found characteristics for the species of quite a different nature from those mentioned above. There is consequently a new direction again opened for the study of these organisms, since the statements hitherto made on this point by different observers are very far from the actual truth.

It is a very generally known phenomenon that fermented liquids become covered with films, and these were for the first time submitted to an exhaustive examination by Hansen. As is known, the films formed by the budding-fungi—*Saccharomyces mycoderma*, *Mycoderma cerevisiæ*, *Mycoderma vini*—have especially attracted attention, and the frequent mention of such films in the literature of our subject has resulted in those consequences, which are also well known in other subjects, namely, they have been spoken of as something well known for so long a time that they are at last accepted as well-proved facts. It now appears that the statements are quite erroneous.

In his latest work Hansen has treated a large number of films, and among several of such forms, the different varieties of "*Saccharomyces mycoderma*"—which have no endogenous spore formation—received the closest attention. According to de Seynes, Reess, and Cienkowski, these varieties of *Sacch. mycoderma* form ascospores; it is, however, very probable that these workers have had to do with impure films containing true Saccharomycetes also. According to Hansen it

is a matter of no little difficulty to ascertain the purity of such a culture, if it is not started with a single cell, since when the *Sacch. mycoderma* is cultivated as a sedimentary yeast, the cells present an entirely different appearance; they are more greatly filled with plasma, whilst the cells of the film, as is known, are poor in plasma, with vacuoles largely developed. Those forms which are usually considered as *Saccharomyces mycoderma*, readily and quickly form films without any marked previous fermentation. On beer and wort these films are grey and dry in appearance; later they become wrinkled and lighter in colour; between the cells air is found freely mixed. The species of *Torula* investigated by Hansen form similar films; the film of *Chalara mycoderma* is, on the contrary, viscous, tough, and feebly lustrous. With *Monilia*, which, as before mentioned, can occur with budding cells, and directly ferments saccharose (cane-sugar), the film formation is peculiar; even during vigorous fermentation there is formed a film of bubbles, which little by little spreads over the whole surface, and sometimes becomes wrinkled. The cells in the flask first sink to the bottom like sedimentary yeast, set up a vigorous fermentation, and again rise with the bubbles of carbonic acid to the surface, where they enter upon a new phase of development. If sterilized lager-beer is infected with this ferment, no fermentation is excited, and only a thin powdery film is developed; under other conditions the ferment forms a white wool-like pellicle resembling that of *Oïdium*.

Films also occur with the true Saccharomycetes, somewhat different, however, from those above mentioned, and the same holds good for some of Pasteur's *Torula* and for *Saccharomyces apiculatus*. According to these experiments the film-formation must not be regarded as something peculiar to certain species, but as a phenomenon common to micro-organisms in general.

With the species of Saccharomyces this formation generally takes place when cultures in wort have been allowed to stand undisturbed for a longer or shorter time at the ordinary temperature; at the end of the primary fermentation small

specks of yeast gradually appear on the surface of the liquid; these coalesce later to figures of various shapes and sizes, to islands, the upward turned portion of which is flat, and the under submerged portion arched. Finally they fuse together to a closely connected, mucilaginous film, usually of a bright greyish-yellow colour, which may be continued to the walls of the vessel as an entire ring. Such a complete film-formation only takes place when the primary fermentation is at an end. If the flask is shaken, single shreds of the film become broken off and sink to the bottom; in this way an entire layer can gradually be heaped up on the bottom, whilst the film is again renewed and presents a marbled appearance, since the newer portions are thin and dark, the older, on the contrary, thick and light.

The conditions, therefore, under which the films can be formed are a free, quiet surface, with direct access to atmospheric air, and a vigorous film-formation therefore presupposes a liberal supply of air. The importance of this latter condition for the formation of films among the Saccharomycetes is shown by the following experiment. Two series of Pasteur's flasks with wort of the same nature and volume were infected with the same species of *Saccharomyces* and given up to the film-formation. One series of flasks was so placed that the bent tube dipped into water, and the carbonic acid evolved then rose in bubbles through the water; the second series was allowed to remain in the ordinary manner without the tubes being dipped into water. The result was that a film was formed in both cases, but more slowly and feebly in the first series of experimental flasks than in the second. From this it follows that a much quicker and more vigorous development takes place in the Chamberland flasks or in ordinary flasks covered with filter-paper than in the Pasteur's flasks with their limited supply of air. The function of the film-formation resembles in this respect the formation of endospores also.

Simultaneously with the formation of a film, a decolourization of the wort begins, and continues until it becomes light-yellow. This reaction takes place most quickly at the higher

temperatures, and occurs most markedly with those species which give rise to the most vigorous film-formation.

The preliminary culture of the cells is the same as that described in connection with the formation of ascospores. The liquid is removed from the growth formed, and fresh sterilized wort is added. A drop of the well-shaken mixture of yeast and wort is transferred to an ordinary flask of about 130 c.c. capacity previously half filled with wort and covered with filter-paper. This operation must be performed with such care that each inoculation remains free from external contamination. Hansen placed flasks prepared in this way at different temperatures, and determined—

1. The limits of temperature for the formation of films;

2. The length of time required for their appearance at different temperatures; and

3. The microscopic appearance of the growths at the different temperatures.

The important point in these new investigations of the six species previously mentioned, lies in the microscopic appearance of the films of these species at the same temperature, and we obtain here again, from a different point of view to that considered in the preceding sub-chapter, a complete investigation on the relation between the interfering factors and the forms, which proves that we have to do with this number (six) of types or species differing in their inner nature.

The investigation of the films took place, unless it is otherwise stated, when they were so far developed that they could just be seen by the naked eye.

A comparison of the isolated results is given in the following short statement:—

Whilst it can be said as a general rule that the cells of the old films are subject to very remarkable changes of form, it appears that the cells of the films of *Sacch. cerevisiæ I.*, *Sacch. Pastorianus II.*, and *Sacch. ellipsoideus II.* in their young state form no mycelium-like colonies; such may, on the contrary, immediately occur with *Sacch. Pastorianus I.* and *III.* and *Sacch. ellipsoideus I.* In this we find a difference in the nature of these species.

At higher temperatures the six species give only very slight points of difference for the examination, since here only *Sacch. cerevisiæ I.* and *Sacch. ellipsoideus II.* differ from the others. It is quite otherwise, however, when we examine the young films at 13–15° C. The two species, *Sacch. Pastorianus II.* and *Sacch. Pastorianus III.*, which are both top-fermentation yeasts, and the cells of which cannot with certainty be distinguished one from another in the ordinary culture, here show entirely different forms; and a similar striking difference is found between the otherwise similar species *Sacch. ellipsoideus I.* and *II.*

An examination of the limits of temperature for the formation of the films shows that for *Sacch. cerevisiæ I.* and *Sacch ellipsoideus I.* these lie within about 38° and 5–6° C.; for the three *Pastorianus* varieties the limits lie between 34° and 3° C.; *Sacch. ellipsoideus II.* has the same lower limit as the last mentioned, its maximum temperature, however, lying at 38–40° C.

The time limits, compared with those formerly given for the ascospore formation, show that in both cases the development takes place more slowly at the lower temperature than at the higher; at temperatures which lie near the minimum and maximum temperatures the film formation was always very feeble and incomplete.

At temperatures above 13° C. the film of *Sacch. ellipsoideus II.* has so quick and vigorous a development, that the flasks with this yeast can be recognized by this alone. Thus, at 22–23° C. it gave a film completely covering the surface at the end of six to twelve days, whilst the five other species at the end of three times that period only gave a film which was generally much more feebly developed. This species and *Sacch. Pastorianus III.* also gave at the room temperature a vigorous film with comparative rapidity, whilst the other species in the same time were still far behind.

As mentioned above, the film-formations have different maximum temperatures. This stands in connection with the fact that the maximum temperature of the species for budding is not the same. It was here proved that budding and fer-

mentation still take place at temperatures above those at which the development of a film no longer occurs. Thus Hansen still observed at 38–40° C. a considerable fermentation and budding of *Sacch. cerevisiæ I.*, *Sacch. ellipsoideus I.*, and *Sacch. ellipsoideus II.*, and at 34° C. of the three species of the group *Sacch. Pastorianus*. We have here new points of difference for distinguishing the species, for whilst the three *Pastorianus* species under these conditions of culture are killed after, at the most, eleven days at 36–38° C., the other three species remain alive after the same time at 38° C. From this we also see that the law formerly enunciated, that the top-fermentation yeasts are able to develop at higher temperatures than the bottom-fermentation species, is not correct, and that there is a relation between the influence of the temperature on the budding and fermentation on the one side, and on the film-formation on the other.

In close connection with the above-mentioned investigation on the changes of form of the cells of the film-formations, stand the brief remarks recently made by Hansen on cultures on solid nutritive surfaces. Of still greater importance for the analysis of yeast may be the differences in the structure of the spores more recently discovered by him in cultures of bottom-fermentation yeasts and wild yeast.

d. The Behaviour of the Saccharomycetes towards the Carbohydrates.

We mentioned in an earlier place that the mould-fungi behave differently towards the carbohydrates. *Monilia candida* directly ferments saccharose (cane-sugar); among the species of *Mucor* we find differences of action, since *Mucor racemosus* first inverts saccharose and then ferments it, whilst the remaining species, described in a former chapter, can only exert their fermentative power after the inversion of this sugar has been brought about in other ways, &c., &c.

Similar special peculiarities are also found among the Saccharomycetes, in which we here include, for the purpose

of review, the so-called *Sacch. apiculatus* and Pasteur's *Torula* forms.

From Hansen's investigations of the behaviour of the Saccharomyces towards the different kinds of sugar, it appears that large specific differences in this respect obtain among the species. The six species treated of on pages 109—124 all secrete invertase; they convert saccharose into invert sugar and then ferment the latter; they ferment dextrose and maltose, but not lactose. The same remarks apply to all the industrial bottom-yeasts which have been at present examined, and, indeed, to all the alcoholic ferments examined by the author. *Sacch. Marxianus* produces no fermentation in maltose and lactose; it inverts saccharose and excites fermentation in solutions of this sugar; dextrose dissolved in yeast-water is also fermented by this ferment. *Sacch. exiguus* behaves towards the sugars exactly as the last-mentioned ferment. *Sacch. membranæfaciens* does not cause an alcoholic fermentation either in beer-wort, or in solutions of saccharose, dextrose, maltose, or lactose; neither is it able to invert saccharose.

Among the ferments resembling the Saccharomyces *Mycoderma cerevisiæ* can bring about no alcoholic fermentation in the four sugars mentioned above, and it is also not capable of inverting saccharose. *Sacch. apiculatus* produces a slight alcoholic fermentation in beer-wort; it does not ferment maltose, and can neither invert nor ferment saccharose.

Hansen formerly assigned three of the *Torula* forms to the alcoholic ferments, and two of these secreted invertase. Since then he has examined some new species of which the first does not ferment maltose, but inverts saccharose, and excites a vigorous fermentation in saccharose—and dextrose—nutritive solutions. The second gives 1 per cent. by volume of alcohol in beer-wort; it does not ferment maltose and saccharose, neither does it invert the latter; it ferments dextrose solutions.

Monilia candida ferments beer-wort and dextrose; in pure maltose solutions it neither grows nor excites fermentation.

The ferment does not secrete the invertive ferment, but directly ferments saccharose.

We may briefly mention here the results of Borgmann's investigation on the chemical characteristics of beer prepared with pure yeast. He employed the pure cultivated yeast from Old Carlsberg, and found that the ratio of glycerine to alcohol was much less in the pure beers than in those brewed with ordinary yeast.

These results, which form the groundwork for further investigations, undoubtedly show that the Saccharomycetes, like the alcoholic ferments in general, also possess in their chemical-physiological activity special characteristics which can only be discovered by pure cultures.

c. Differences in the Species judged from their Properties in Practice.

Since pure cultures of the same species have been already tested, according to Hansen's directions, on a large scale in the most diverse places, it no longer admits of doubt that the different properties of the pitching yeast as regards breaking, brightening power, attenuation, resistance to yeast-turbidity, &c., &c., must be referred to a very great extent to the variety or race of the yeast itself, and must thus constitute one part of the indwelling characters which should be determined when a selection is made.

Hansen also directed attention to the fact that among the so-called "wild" species of Saccharomycetes very decided peculiarities in this direction existed, since he found groups which produced diseases in beer, whilst others proved themselves harmless after prolonged practical experiments. Among the first-mentioned, some possess the peculiarity of giving a bitter taste to beer, others cause yeast-turbidity—here again, characteristics which are so much part of the species that they serve to distinguish them from others. But we will treat more fully of this point later.

Before we conclude our *résumé* of Hansen's investigations,

it will be advisable to mention here his studies on the gelatinous formation shown by yeast cells.

Under certain not yet sufficiently known conditions, the colonies of yeast cells produced by budding may unite to irregularly sized masses, which sink to the bottom more quickly than the single yeast cells. This undoubtedly stands in connection with a side of the development of yeast cells which Hansen discovered in 1884. He found that not only the Saccharomycetes, but also other budding-fungi are able to separate a gelatinous network, consisting of threads and plates, and within which the cells lie embedded. If, for example, a somewhat thick brewery yeast be placed in a glass and allowed to remain covered up in such a manner that it slowly dries up, and then a trace of this yeast mixed with water, the network is very clearly shown. The formation also occurs in the gypsum and gelatine cultures. I have myself very frequently observed this remarkable formation, after Hansen had made its nature clear to me, in the samples of yeast which are sent to my laboratory in filter-paper in envelopes.* It was also found by Hansen in the film-formations of nearly all species. A general microscopic examination of the pitching yeast in a brewery did not show this formation; with the help of staining, however, it was rendered very evident. When the yeast was repeatedly washed, it was no longer possible to detect the network by staining; if, however, the water was removed, and the mass of cells allowed to remain for some time, the gelatinous masses were easily made visible in a similar preparation. By varying the conditions of nutriment of the cells, the development could be hastened or hindered, the chemical composition altered. The whole matter reminds one of the zooglœa formation of bacteria.

As an introduction to the systematic description of the

* This method of preserving a sample of yeast for some time is very convenient. A small piece of filter-paper is quickly passed several times through the flame, a few drops of yeast are poured on it, and it is folded together, after which it is wrapped in several more pieces which have been similarly treated.

separate species of *Saccharomyces*, we give in the following a general description of the *Saccharomyces* cell.

The microscopic picture of a yeast cell as it appears most frequently in a fermenting liquid is a spherical or oval figure, which by the swelling out of its wall gives rise to one or more buds, these sooner or later separating themselves from the mother cell. This cell is consequently surrounded by a membrane, which in the different stages of development of the cell can become somewhat different, but seldom in an evident manner. Quite otherwise, however, is the behaviour of the contents of this cell. The most simple picture of these contents is obtained when we observe the cell in its most vigorous state of growth; the cell-contents then consist of clear and homogeneous plasma. With the continued multiplying—and fermentative—activity different bodies occur in this plasma; partly clear portions which are full of cell-sap (vacuoles), partly larger and smaller bodies, of which some may be regarded as fat-globules, whilst others appear of a similar nature to the plasma. This granular condition of the plasma gradually increases, and at a very advanced stage of the fermentation, when the cell has nearly arrived at a resting condition, the plasma may be reduced to a very fine layer on the inner side of the cell, whilst the remaining space is filled by a large vacuole, which contains numerous large and small bodies, very many of which are fatty. If such cells are again brought into a liquid capable of fermentation, they present a highly characteristic picture during the short period which precedes the microscopic phenomena of fermentation. The granulations disappear, and numerous fine threads of plasma occur in the clear cell-sap, and gradually mark out rounded vacuoles; finally these disappear, and the cell is again filled with a clear, homogeneous plasma.



As in the majority of vegetable cells, we also find in the yeast cell a cell-nucleus (first discovered by Schmitz), which can be detected by staining with osmic acid, picric acid, or hæmatoxylin. According to Hansen this cell-nucleus is either spherical or disc-like; he found it in the old film-formations of Saccharomycetes cells, which clearly showed

the cell-nucleus without any preparation. Of the endogenous spores, as well as of the gelatinous formation, we have already spoken.

CLASSIFICATION OF THE GENUS SACCHAROMYCES.

BUDDING-FUNGI, FOR THE MOST PART WITHOUT A MYCELIUM, the individual species of which occur with cells of different form and size. Under certain treatment, also sometimes without previous preparation, cell-nuclei occur. Under certain conditions the cells develop ENDOGENOUS SPORES; the germinating spores grow to budding cells. Number of spores 1—10, most frequently 1—4. The cells separate, under favourable conditions, a gelatinous network in which complex cells are embedded.

The whole of the species excite an alcoholic fermentation.

SACCHAROMYCES CEREVISIÆ I.—HANSEN.

(Figs. 23—25.)

Top-fermentation yeast.

The growth of sedimentary yeast (Fig. 23), developed in wort, consists principally of large round or oval cells; true elongated cells do not occur.

The old English top-fermentation yeast employed in the breweries of Edinburgh and London.

Mode of the ascospore formation (Fig. 22, 1):—

At	37·5°	C. no ascospores are developed.	
,,	36—37	,, the first appearance takes place after	29 hours
,,	35	,, ,, ,, ,, ,,	25 ,,
,,	33·5	,, ,, ,, ,, ,,	23 ,,
,,	30	,, ,, ,, ,, ,,	20 ,,
,,	25	,, ,, ,, ,, ,,	23 ,,
,,	23	,, ,, ,, ,, ,,	27 ,,
,,	17·5	,, ,, ,, ,, ,,	50 ,,
,,	16·5	,, ,, ,, ,, ,,	65 ,,
,,	11—12	,, ,, ,, ,, ,,	10 days
,,	9	,, no ascospores are developed.	

Spores very refractive. Wall of the spores very distinct. Size of the spores 2·5—6 μ.

Film-formation :—

	Temp.							Time
At	38°	C.	no film-formation occurs.					
,,	33—34	,,	feebly developed flecks of film are seen after					9—18 days
,,	26—28	,,	,,	,,	,,	,,		7—11 ,,
,,	20—22	,,	,,	,,	,,	,,		7—10 ,,
,,	13—15	,,	} (Fig. 24) {		,,	,,		15—30 ,,
,,	6— 7	,,	} (Fig. 24) {		,,	,,		2— 3 months
,,	5		no film-formation occurs.					

Microscopic appearance of the cells in the films :—

At 20—34° C. : Colonies frequent ; sausage-like and oddly-shapen cells occur.

At **15—6**° C. (Fig. 24): The majority of the cells the same form as the parent cells ; isolated irregular cells.

In old cultures of the films all forms of cells occur, including very greatly elongated, mycelium-like cells (Fig. 25).

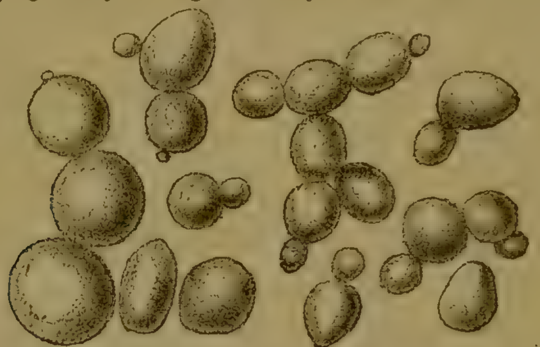


FIG. 23.—SACCH. CEREVISIÆ I.—HANSEN.
Sedimentary forms (after Hansen).

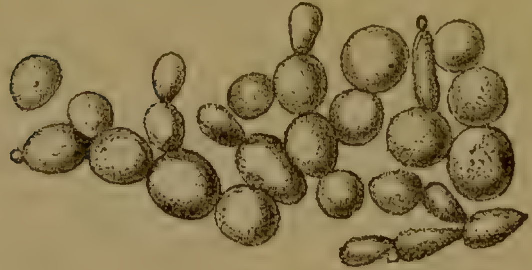


FIG. 24.—SACCH. CEREVISIÆ I.—HANSEN.
Film forms at 15—6° C. (after Hansen).



Fig. 25.—Sacch. cerevisiæ I.—Hansen.
Forms of Cells in Old Cultures of the Films (after Hansen).

SACCHAROMYCES PASTORIANUS I.—HANSEN.

(Figs. 26, 27.)

Bottom-fermentation yeast.

Sedimentary forms grown in wort: Elongated sausage-shaped cells predominate; large and small oval and round cells also occur (Fig. 26).

Frequently occurs in the air of fermenting rooms. Gives a strong, bitter taste to beer.



✓30/10/91

FIG. 26.—SACCH. PASTORIANUS I.—HANSEN.
Sedimentary forms (after Hansen).

Mode of the ascospore formation (Fig. 22, 2):—

	Temperature			Time
At	31·5°	C.	no ascospores are developed.	
,,	29·5—30·5	,,	the first appearance takes place after	30 hours
,,	29	,,	,, ,, ,, ,,	27 ,,
,,	27·5	,,	,, ,, ,, ,,	24 ,,
,,	23·5	,,	,, ,, ,, ,,	26 ,,
,,	18	,,	,, ,, ,, ,,	35 ,,
,,	15	,,	,, ,, ,, ,,	50 ,,
,,	10	,,	,, ,, ,, ,,	89 ,,
,,	8·5	,,	,, ,, ,, ,,	5 days
,,	7	,,	,, ,, ,, ,,	7 ,,
,,	3—4	,,	,, ,, ,, ,,	14 ,,
,,	0·5	,,	no ascospores are developed.	

Size of the spores 1·5—5 μ.

6/11/91 - At 25°C - a large majority of cells in growths on gypsum with cultivation of one day old, in 40-42 hours + 5 ... 15-

Film-formation :—

At 34° C. no film-formation occurs.
,, 26—28 ,, feebly developed flecks are seen after 7—10 days.
,, 20—22 ,, ,, ,, ,, ,, 8—15 ,,
,, 13—15 ,, } (Fig. 27) { ,, ,, 15—30 ,,
,, 6—7 ,, } (Fig. 27) { ,, ,, 1—2 months.
,, 3—5 ,, ,, ,, ,, ,, 5—6 ,,
like Fig. 27, but without the large colonies.
,, 2—3 ,, no film-formation occurs.

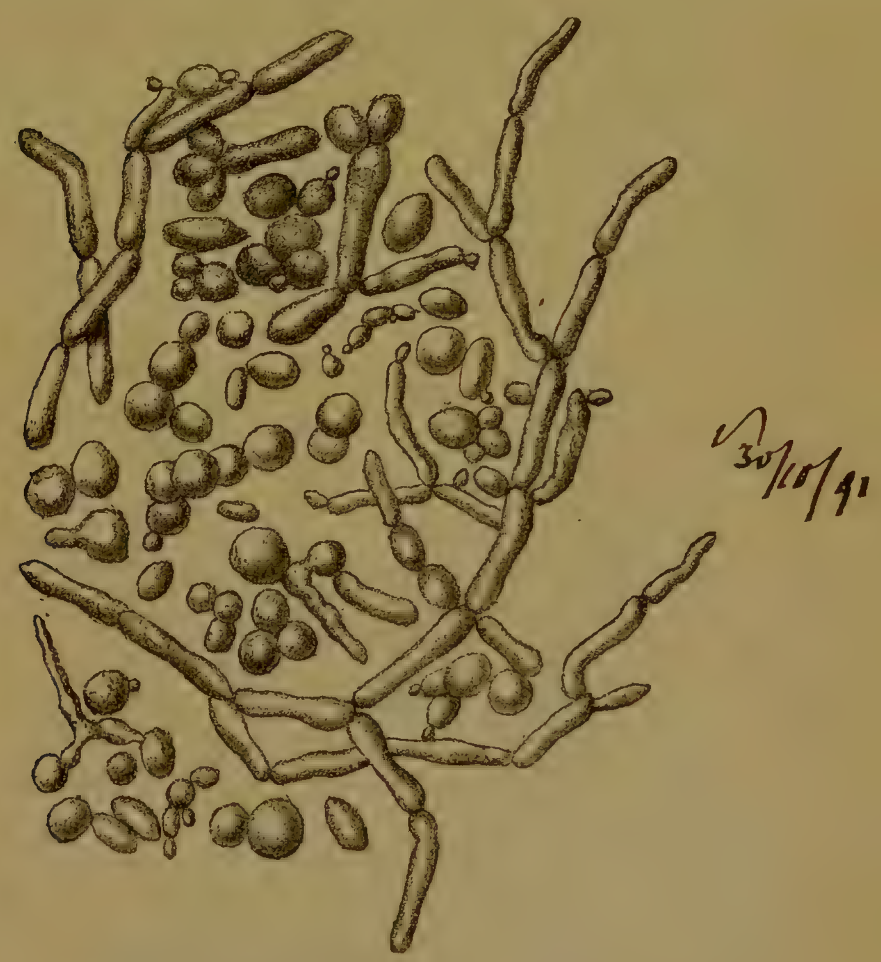


FIG. 27.—SACCH. PASTORIANUS I.—HANSEN.
Film forms at 13-15° C. (after Holm in Hansen's Memoir).

Microscopic appearance of the cells in the films :—

At 20—28° C. : Nearly the same forms as in the sedimentary yeast.

At **13--15**° C. : Strongly developed mycelium-like colonies of very elongated, sausage-shaped cells (Fig. 27), frequent.

In old cultures of the films the cells are smaller than in the sedimentary yeast; there occur very irregular, sometimes almost thread-like, cells.

Saccharomyces Pastorianus II.—Hansen.

(Figs. 28, 29.)

Feeble top-fermentation yeast.

Sedimentary forms grown in wort: Elongated, sausage-shaped cells predominate; large and small oval and round cells are also present (Fig. 28).



Fig. 28.—Sacch. Pastorianus II.—Hansen.
Sedimentary forms (after Hansen).

Occurred frequently in Hansen's analyses of the air of breweries; appears to belong to the species which give rise to no disease in beer.

Mode of the ascospore formation (Figs. 22, 23) :—

At 29°	C. no ascospores are developed.	
,, 27—28	,, the first appearance takes place after	34 hours.
,, 25	,, ,, ,, ,, ,,	25 ,,
,, 23	,, ,, ,, ,, ,,	27 ,,
,, 17	,, ,, ,, ,, ,,	36 ,,
,, 15	,, ,, ,, ,, ,,	48 ,,
,, 11·5	,, ,, ,, ,, ,,	77 ,,
,, 7	,, ,, ,, ,, ,,	7 days.
,, 3—4	,, ,, ,, ,, ,,	17 ,,
,, 0·5	,, no ascospores are developed.	

Sizes of the spores 2—5 μ.



Fig. 29.—Sacch. Pastorianus II.—Hansen.
Film forms at 15—3° C. (after Holm in Hansen's Memoir).

Film-formation :—

At 34°	C. no film-formation occurs.	
,, 26—28	,, feebly developed flecks are seen after	7—10 days.
,, 20—22	,, ,, ,, ,, ,,	8—15 ,,
,, 13—15	,, ,, ,, (Fig. 29) ,, ,,	10—25 ,,
,, 6—7	,, (Fig. 29) ,, ,,	1—2 months.
,, 3—5	,, ,, ,, (Fig. 29) ,, ,,	5—6 ,,
,, 2—3	no film-formation occurs.	

Microscopic appearance of the cells in the films :—

At 20—28° C. : Nearly the same forms as in the sedimentary yeast; with the addition of irregular sausage-shaped cells.

At **15**—**3**° C. : A predominance of oval and round cells.

In old cultures of the films the cells are smaller than in the sedimentary yeast; there are also found very irregular, sometimes almost thread-like, cells.

SACCHAROMYCES PASTORIANUS III.—HANSEN.

(Figs. 30, 31.)

Top-fermentation yeast.

Sedimentary forms grown in wort: Elongated, sausage-shaped cells predominate; large and small oval and round cells are also present (Fig. 30).

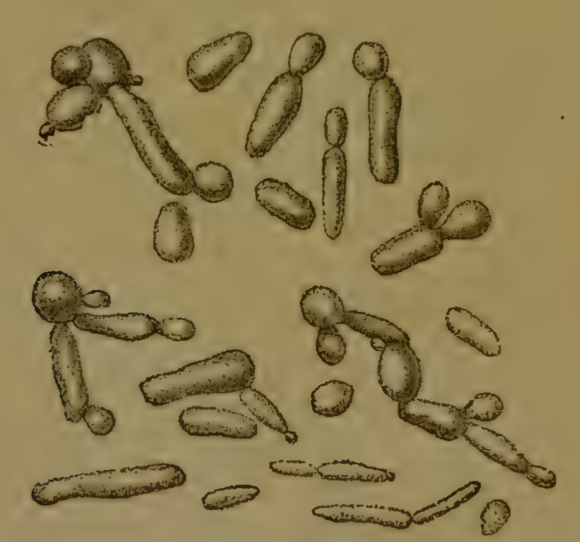


FIG. 30.—SACCH. PASTORIANUS III.—HANSEN.
Sedimentary forms (after Hansen).

Was separated from bottom-fermentation beer suffering from yeast-turbidity, and has been shown by Hansen to be one of the species causing yeast-turbidity.

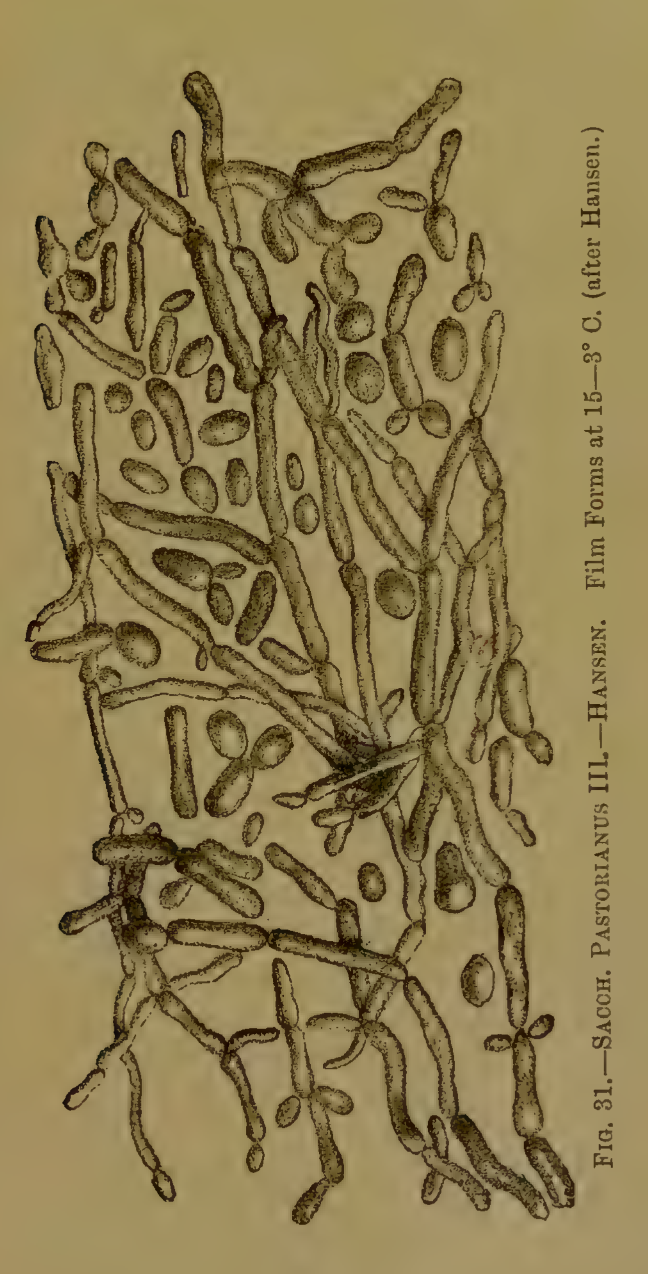


Fig. 31.—Sacch. Pastorianus III.—Hansen. Film Forms at 15—3° C. (after Hansen.)

Mode of the ascospore formation (Fig. 22, 4):—

At 29° C.	no ascospores are developed.	
,, 27—28 ,,	the first appearance takes place after	35 hours.
,, 26·5 ,,	the first appearance takes place after	30 ,,
,, 25 ,,	the first appearance takes place after	28 ,,
,, 22 ,,	the first appearance takes place after	29 ,,
,, 17 ,,	the first appearance takes place after	44 ,,
,, 16 ,,	the first appearance takes place after	53 ,,
,, 10·5 ,,	the first appearance takes place after	7 days.
,, 8·5 ,,	the first appearance takes place after	9 ,,
,, 4 ,,	no ascospores are developed.	

Size of the spores 2—4 μ.

Film-formation :—

At 34° C.	no film-formation occurs.	
,, 26—28 ,,	feebly developed flecks are seen after	7—10 days.
,, 20—22 ,,	feebly developed flecks are seen after	9—12 ,,
,, 13—15 ,,	feebly developed flecks (Fig. 31) are seen after	10—20 ,,
,, 6— 7 ,,	feebly developed flecks (Fig. 31) are seen after	1— 2 months
,, 3— 5 ,,	feebly developed flecks (Fig. 31) are seen after	5— 6 ,,
,, 2— 3 ,,	no film-formation occurs.	

Microscopic appearance of the cells in the films :—

At 20—28° C. : Nearly the same forms as in the sedimentary yeast.

At **15—3**° C. : Strongly developed colonies of elongated, sausage-shaped, or thread-like cells, which very closely resemble a mycelium in appearance.

In old cultures of the films the cells are of the same form as at 15—3° C., and often still thinner and more thread-like.

Saccharomyces ellipsoideus I.—Hansen.

(Figs. 32, 33.)

Bottom-fermentation yeast.

Sedimentary forms grown in wort ; oval and round cells predominate ; sausage-shaped cells rare (Fig. 32).

On the surface of ripe grapes.

Mode of ascospore formation (Fig. 22, 5):—

At	32·5° C.	no ascospores are developed.	
,,	30·5—31·5	,, the first appearance takes place after	36 hours.
,,	29·5	,, ,, ,, ,, ,,	23 ,,
,,	25	,, ,, ,, ,, ,,	21 ,,
,,	18	,, ,, ,, ,, ,,	33 ,,
,,	15	,, ,, ,, ,, ,,	45 ,,
,,	10·5	,, ,, ,, ,, ,,	4·5 days.
,,	7·5	,, ,, ,, ,, ,,	11 ,,
,,	4	,, no ascospores are developed.	

Size of spores 2—4 μ.



FIG. 32.—SACCH. ELLIPSOIDEUS I.—HANSEN. Sedimentary forms (after Hansen).

Film-formation :—

At	38° C.	no film-formation occurs.	
,,	33—34	,, feebly developed flecks are seen after	8—12 days.
,,	26—28	,, ,, ,, ,, ,,	9—16 ,,
,,	20—22	,, ,, ,, ,, ,,	10—17 ,,
,,	13—15	,, (Fig. 33) ,, ,, ,,	15—30 ,,
,,	6— 7	,, ,, ,, ,, ,,	2—3 months.
,,	5	,, no film-formation occurs.	

Microscopic appearance of the cells in the films :—

At 20—34° C. and 6—7° C.: Cells smaller and proportionally more sausage-shaped than the sedimentary yeast.

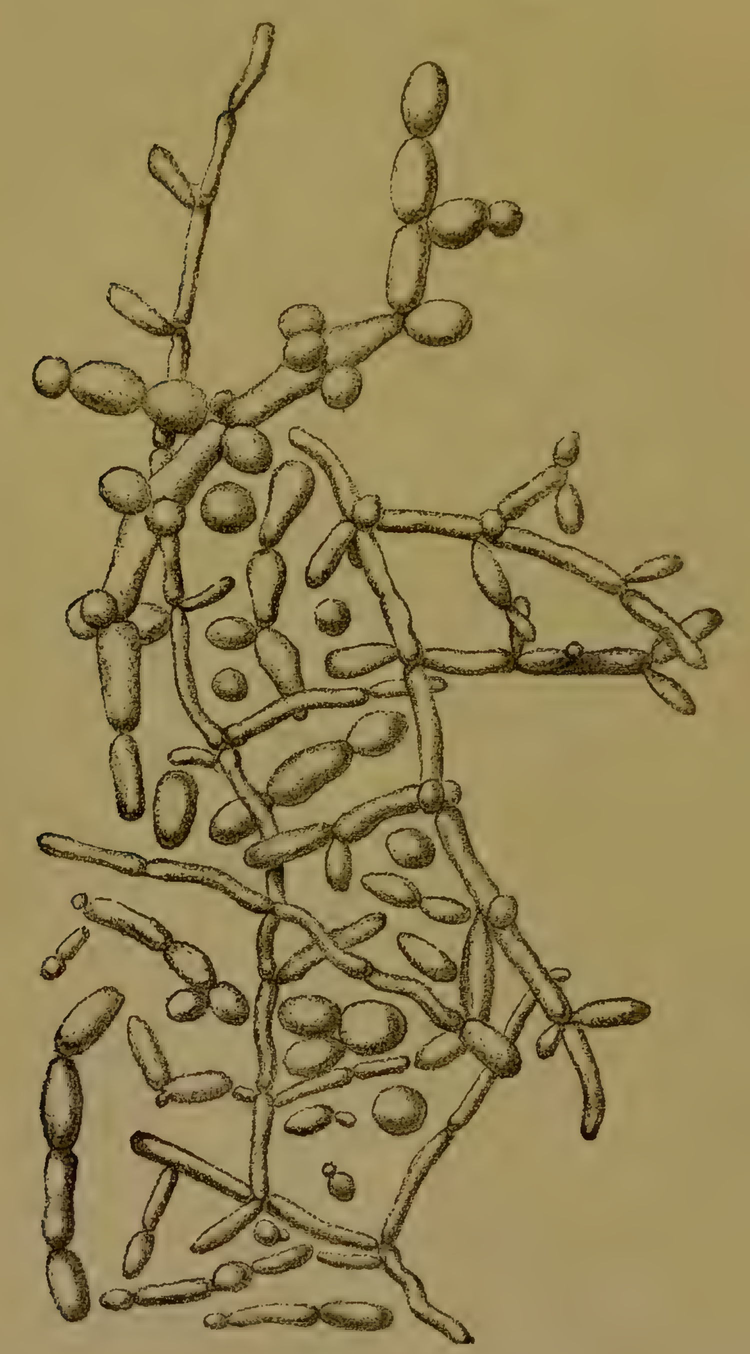


FIG. 33.—SACCH. ELLIPSOIDEUS I.—HANSEN.
Film forms at 13—15° C. (after Holm in Hansen's Memoir)

At **13—15°** C.: Freely branched and well-developed colonies of short or long sausage-shaped cells, often with forked branches (Fig. 33).

In old cultures of the films the cells are the same as at 13—15° C.

SACCHAROMYCES ELLIPSOIDEUS II.—HANSEN.

(Figs. 34, 35.)

Bottom-fermentation yeast.

Sedimentary forms grown in wort: Oval and round cells predominate; sausage-shaped cells rare (Fig. 34).

Separated from a beer suffering from yeast-turbidity; a species capable of causing yeast-turbidity.

X Mode of ascospore formation (Fig. 22, 6):—

At	35° C.	no ascospores are developed.	
,,	33—34 ,,	the first appearance takes place after	31 hours.
,,	33 ,,	,, ,, ,, ,,	27 ,,
,,	31·5 ,,	,, ,, ,, ,,	23 ,,
,,	29 ,,	,, ,, ,, ,,	22 ,,
,,	25 ,,	,, ,, ,, ,,	27 ,,
,,	18 ,,	,, ,, ,, ,,	42 ,,
,,	11 ,,	,, ,, ,, ,,	5·5 days.
,,	8 ,,	,, ,, ,, ,,	9 ,,
,,	4 ,,	no ascospores are developed.	

Size of the spores 2—5 μ.

Film-formation:—

At	40° C.	no film-formation occurs.	
,,	36—38 ,,	feebly developed flecks are seen after	8—12 days.
,,	33—34 ,,	,, ,, ,, ,, ,,	3—4 ,,
,,	26—28 ,,	,, (Fig. 35) ,, ,, ,, ,,	4—5 ,,
,,	20—22 ,,	,, (Fig. 35) ,, ,, ,, ,,	4—6 ,,
,,	13—15 ,,	(Fig. 35) ,, ,, ,,	8—10 ,,
,,	6—7 ,,	,, (Fig. 35) ,, ,, ,, ,,	1—2 months.
,,	3—5 ,,	,, (Fig. 35) ,, ,, ,, ,,	5—6 ,,
,,	2—3 ,,	no film-formation occurs.	

x P25 – A majority of the cells with ascospores – appear [illegible] uniformly 1 ascospore – in 40-42 hours + 5 hours [illegible] table E [illegible]

Microscopic appearance of the cells in the films :—

At all temperatures the same forms as in the sedimentary yeast; at 15° C. and below, only slightly more elongated (Fig. 35).

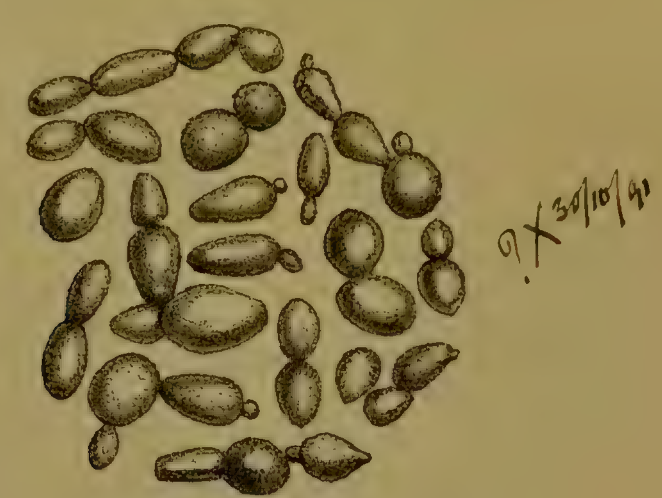


FIG. 34.—SACCH. ELLIPSOIDEUS II.—HANSEN.
Sedimentary forms (after Hansen).

In old cultures of the films there are colonies of short and long sausage-shaped cells, often with forked branches.



FIG. 35.—SACCH. ELLIPSOIDEUS II.—HANSEN.
Film forms at 28—3° C. (after Hansen).

From the foregoing the following differential characteristics for these six species can be deduced :—

Saccharomyces cerevisiæ I.

A top-fermentation yeast employed in practice.

Develops ascospores at temperatures between 11° and 37° C. (under the conditions described on pp. 94-99)

Film-formation at 13—15° C. (under the conditions given on pp. 99-104): an immense majority of the cells formed like those of the sedimentary yeast.

Saccharomyces Pastorianus I. Bottom-fermentation form. Gives a bitter taste to beer.

Develops ascospores at temperatures between 3° and 30·5° C.

Film-formation at 13—15° C.: tolerably frequent, well-developed, mycelium-like colonies of very elongated, sausage-shaped cells.

Saccharomyces Pastorianus II. Top-fermentation form. Produces no disease in beer.

Develops ascospores at temperatures between 3° and 28° C.

Film-formation at 13—15° C.; oval and round cells predominate.

Saccharomyces Pastorianus III. Top-fermentation form. Causes yeast-turbidity.

Develops ascospores at temperatures between 8·5° and 28° C.

Film-formation at 13—15° C.: well-developed colonies of sausage or thread-shaped, mycelium-like cells.

Saccharomyces ellipsoideus I. Bottom-fermentation form. The yeast of grapes.

Develops ascospores at temperatures between 7·5° and 31·5° C.

Film-formation as 13—15° C.: richly branched and well-developed colonies of short and long cells, branches often forked.

Saccharomyces ellipsoideus II. Bottom-fermentation form. Causes yeast-turbidity.

Develops ascospores at temperatures between 8° and 34° C.

Film-formation at 13—15° C.: essentially like the sedimentary forms.

SACCHAROMYCES MARXIANUS.—HANSEN.

This species, under ordinary conditions, develops in beer-wort small oval and egg-shaped cells, among which are quickly found sausage-shaped and elongated cells frequently combined in colonies. When the culture has remained quiet for some time, there appear small viscous bodies, which partly swim in the liquid and partly sink to the bottom of the vessel; the cells of these bodies resemble the cells of the films of the six species of Saccharomyces described in the preceding pages. The cells of this species develop ascospores, but not to any great extent; these spores are partly round and oval, and partly kidney-shaped. This species gives, even after standing two to three months, only traces of films with oval and short sausage-shaped cells.

This yeast belongs to those species which, under certain conditions of culture on solid nutritive substances, give a mycelium.

In beer-wort it forms only 1 to 1·3 per cent. by volume of alcohol, and is not able to ferment maltose. It inverts saccharose, and produces large quantities of alcohol in nutritive solutions, both with invert-sugar and with dextrose.

SACCHAROMYCES EXIGUUS.—REESS.

Saccharomyces exiguus develops under the ordinary conditions a growth showing the same cell-forms which are described by Reess under this name; Hansen therefore gives this name to the ferment described in the following, although it cannot be determined whether Reess had the same ferment.

Small yeast-cells resembling those described by him may be formed by every species of Saccharomyces.

This ferment forms endospores with difficulty; it also gives only a feeble film, but a copious yeast-ring. The cells of the film are short, and sausage-like, with the smaller forms very numerous. Hansen found this species in bakers' yeast. It gives no mycelium-like colonies when cultivated in wort, neither does it form mycelia on solid nutritive material.

In beer-wort it yields about 1 per cent. of alcohol, which stands in connection with its inability to ferment maltose. It inverts saccharose and excites a vigorous alcoholic fermentation in saccharose and dextrose solutions.

SACCHAROMYCES MEMBRANÆFACIENS.—HANSEN.

This species quickly forms on the surface of beer-wort a bright yellow, felted, thick film composed of sausage-like and long cells, partly united in colonies, partly isolated. It very freely develops ascospores, both under the ordinary cultural conditions as well as in the films. On nutritive gelatine it forms dull grey flecks, which often appear with a feeble reddish flush, and are usually considerably drawn out, rounded and wrinkled; the colonies, however, which are covered with gelatine, present an entirely different appearance. The nutritive gelatine is liquefied.

This yeast does not cause fermentation either in beer-wort or in solutions of saccharose, maltose, dextrose, or lactose; neither does it invert saccharose.

Hansen found this ferment in a gelatinous mass, which together with various fungi had formed round the roots of an elm-tree.

Sacch. membranæfaciens is the only Saccharomycetes at present known which does neither excite fermentation nor secrete invertase.

SACCHAROMYCES MINOR.—ENGEL.

According to the description of Engel the vegetative cells

are completely spherical, as much as 6 μ in diameter, combined in chains or in colonies consisting of a few cells (6—9). Spore forming cells 7—8 μ, containing 2—4 spores of 3 μ diameter.

This organism is, according to the above-mentioned author, the active ferment in the fermentation of bread. Compare, however, for black bread the observations made by Laurent (page 50).

Saccharomyces conglomeratus.—Reess.

This is described by Reess as follows:—"Budding-cells round, of 5—6 μ in diameter, combined in clusters which have their origin from the axes of two old cells, before these further bud in the direction of their common longitudinal axes to a series of cells, almost simultaneously throwing out several buds as branches. The asci very frequently remain combined in pairs or with a vegetative cell, spores 2—4, giving rise, on germination, to fresh clusters. On decaying grapes and in wine-yeast at the commencement of the fermentation. Fermentative action doubtful."

In Hansen's cultures of film-formations of the Saccharomycetes there occurred cell-colonies of the above-mentioned appearance in the old films with all the six species investigated by Hansen. And since this investigator never found a definite species which could be identified with Reess' *Sacch. conglomeratus*, he is inclined to think that these cell-colonies of the different Saccharomycetes are identical with this species.

The different races or species of yeast may be divided into two groups according to their form of fermentation—bottom-yeast and top-yeast. In spite of many assertions to the contrary it has hitherto been impossible to bring about an actual conversion of top-yeast into bottom-yeast, and *vice versâ*. According to the investigations of Hansen and Kühle it is easily possible to produce transitory top-fermentation phenomena with a bottom-fermentation yeast; these, however,

quickly disappear with the progressive development of the yeast. Therefore, when it is stated that bottom-yeast, for instance, can be converted into top-yeast by continued cultivation at an elevated temperature, we must first assume that the bottom-yeast employed was impure and had contained admixed top-yeast, which slowly developed by cultivation at an elevated temperature at the expense of the bottom-yeast, until it finally constituted the chief portion of the yeast.*

In addition to the beer top-yeast, there are also certain races of top-yeast employed in distilleries and yeast manufactories. The investigation of these races is not so far advanced as that of the races of beer-yeast. Bêlohoubek, Schumacher, and Wiesner, have carried out microscopical and chemical investigations, and the first-named author's "Studien über Presshefe" (Prague, 1876) especially contains accurate descriptions of the appearance under the microscope of ordinary pressed-yeast in the different stages of its growth,

* A preliminary grouping in a practical direction of the different races of top-fermentation and bottom-fermentation beer-yeast, which have been prepared in a pure state in the laboratories of Hansen and myself, is as follows :—

A.—Bottom-Fermentation Races.

1. Brightening very quickly and attenuating slightly in fermenting vessel; beer holding a strong head. The beer is not protected from yeast-turbidity by long storage. Such races are only suitable for draught-beer.

2. Brightening fairly quickly and not attenuating greatly; beer holding a strong head; these races settle very solid in the fermenting vessels. The beer is not particularly resistive to yeast-turbidity. Suitable for draught-beer and partly for lager-beer.

3. Races which brighten slowly and attenuate greatly; the beer has a fine taste and smell; the yeast settles less solidly in the fermenting vessels. The beer is very resistive to yeast-turbidity. Suited for lager-beer and especially for export-beer, when this is not pasteurized or treated with antiseptics.

B.—Top-Fermentation Races.

1. Slightly attenuating, quickly brightening. The beer has a sweet taste.

2. Greatly attenuating, quickly brightening. More pronounced taste.

3. Slowly brightening, giving a normal after-fermentation. Taste more wine-like. The beer resistive to yeast-turbidity.

and observations on the microscopic characteristics of the more or less good condition of the manufactured yeast, so far as it can be judged from the contents of the cell. During disintegration the ideal cells of yeast change in colour and in the consistence of the plasma; this becomes gradually darker, more limpid, the vacuoles are larger, and the sharp outlines between vacuoles and plasma disappear little by little, the plasma separates itself from the cell-wall, and finally collects together in irregular masses in the fluid of the cell; finally these also disappear and at last the wall is dissolved. In addition there occur, according to this author, cells in pressed yeast which suddenly develop a larger number of small vacuoles; these "abnormal vacuolar" cells quickly die.

UNCLASSIFIED BUDDING-FUNGI.

("*Torula*," "*Saccharomyces apiculatus*," "*Mycoderma cerevisiæ*," *&c.*, *&c.*)

We give a review of such of these ferments as are of importance in the fermentation industries, and which so far agree with the Saccharomycetes that they multiply by budding; only under exceptional circumstances does a mycelium occur with these species. They may be all distinguished from the Saccharomycetes by the want of the endogenous spore formation, which is characteristic of all the latter.

Properly speaking, the *Torula*-forms investigated by Hansen and which give a mycelium must be regarded as belonging to the mould-fungi. Since, however, it has not yet been systematically determined to what class of mould-fungi they belong, these species are, on practical grounds, placed in the present chapter.

TORULA.

The yeast-like forms which Pasteur figured and described under the name *Torula*, are widely distributed, and, therefore,

frequently occur in the physiological analyses connected with fermentation. They occur both with spherical and with more or less elongated forms, and differ, as was first pointed out by Hansen, from the Genus Saccharomycetes in that they are not able to form endogenous spores. They multiply in the majority of cases only by budding, in some few cases also by the formation of mycelia.

Hansen observed seven different species :

The first occurs in wort, either isolated or in colonies consisting of few cells. Some cells have a large vacuole in the centre, sometimes with a small, very refractive granulation. The size of the cells is very varied (1·5 to 4·5 μ). The species secretes no invertase, and causes a scarcely perceptible alcoholic fermentation in beer-wort.

The second species under the same conditions has larger cells than the first (3 to 8 μ). They resemble the foregoing, with the exception that the cells, cultivated in wort, often exhibit a very granulated cell-contents.

The third species, which, under the microscope, resembles the last mentioned, produces under the same conditions as much as 0·88 per cent. by volume of alcohol ; it gives a marked "head" with evolution of carbonic acid. It cannot, however, invert cane-sugar.

The fourth species (2 to 6 μ) is able to invert cane-sugar, and produces in wort, with great frothing, a little more than 1·0 per cent. by volume of alcohol ; it does not, however, ferment maltose.

The fifth species, which resembles the first so far as form and size of the cells are concerned, develops a homogeneous, dull grey film on wort and on yeast-water at the ordinary room temperature ; the film is also formed on lager beer and liquids containing as much as 10 per cent. of alcohol. It inverts saccharose, and gives rise to a feeble film on the inverted solution. It excites, however, no appreciable alcoholic fermentation.

A sixth species, which Hansen later separated from the air, forms small round, oval cells that excite a considerable fermentation in beer-wort, and produce 1·3 per cent. by volume

of alcohol. It inverts saccharose, and yields 5·1 to 6·2 per cent. of alcohol in the inverted solution with yeast-water after fourteen days at 25° C. In dextrose nutritive solutions it produces a still higher alcoholic percentage.

A seventh species, examined by the same investigator, was found in the earth under grape-vines. The cells are round and oval. In old films the cells are frequently larger, irregular and elongated sausage-shaped. In beer-wort this species forms 1 per cent. of alcohol; it does not ferment maltose and saccharose, and does not secrete invertase. In 10 to 15 per cent. solutions of dextrose in yeast-water it gives 4·6 to 4·5 per cent. of alcohol after fifteen days at 25° C.; and after one month, 4·4 to 4·7 per cent.

These seven species cannot be differentiated by the microscope alone, or distinguished from the round cells of the species of Saccharomyces. Pasteur separated the Torula forms from the group of the Saccharomycetes, because they produced only a feeble alcoholic fermentation. According to the above researches, however, there also exist species with distinct fermentative activity.

Hansen, with some probability, assumes that they are related to the higher fungi, and has, as mentioned above, observed in a few cases the development of a mycelium during the cultivation experiments.

Saccharomyces apiculatus.

(Fig. 36).

As already remarked, the name of this ferment is not correct according to the opinions now held, since only those budding-fungi which produce endogenous spores can be considered as belonging to the Saccharomycetes; this power is not possessed by our ferment. It may, however, be correct, as Hansen suggested, to provisionally retain the old generic name until a more comprehensive scheme is prepared for the whole matter.

As is known, this ferment gave rise to one of the finest and most thorough biological investigations of our time, since Hansen was enabled by the work of many years to determine both its habitat in nature and also its regular migrations during the different seasons of the year. The reason that this species was selected for investigation was because all the other species occur with extremely diverse and undeterminate forms, which makes the study of their occurrence in different localities in the highest degree difficult, whilst this ferment can be recognized with certainty by its form, since it always occurs in cultures with citron-like cells; this is the typical form of the species.

The ferment occurs abundantly in wine-yeast, especially in the first stages of the fermentation, also in the Belgian



FIG. 36.—SACCHAROMYCES APICULATUS.

A. Forms of growth; *n*. Sacch. cerevisiæ (for comparison). *B*. Stages of development of *Saccharomyces apiculatus; a*. 2½ hours; *b*. 3¼ hours; *c*. 4 hours (all cells have taken the citron form); *d*. 5 hours (the continued development of the two cells shown on the right at *c*); *e*. 5½ hours. (After Hansen.)

spontaneously-fermented beer, and freely on ripe, sweet, succulent fruit.

If a little of such a growth is brought in a drop of nutritive liquid under the microscope, the development of the ferment can be followed. This is, as was first pointed out by Hansen, very peculiar (compare Fig. 36). It is seen that the buds formed from the typical citron-like cells may be either citron-shaped or oval; it is also seen that the oval cells must first form one or more buds before they are able to take the

citron form, and finally that the citron-like shape of a cell may be lost during the budding. Under other conditions the cells may take entirely different forms: sausage-shaped, half-moon-shaped, bacterium-like, &c. Is there any order in this apparent confusion of forms? In the foregoing it was shown that the ferment can separate two kinds of buds, and that the oval buds must give rise to one or more buds before they can take the normal form. The question then is: Under what conditions are the two kinds of buds developed? By cultural experiments it was shown that the citron-shaped buds are especially developed in the first stages of the culture, afterwards, however, they are crowded out by the oval forms.

We will now give a further description of the ferment from a physiological and biological standpoint.

Sacch. apiculatus is a bottom-fermentation yeast which is capable of exciting alcoholic fermentation in beer-wort; the alcoholic fermentation in this liquid is, however, feeble, since *Sacch. apiculatus* only produces 1 per cent. by volume of alcohol, whilst *Sacch. cerevisiæ* (bottom-yeast) under the same conditions gives 6 per cent. by volume of alcohol. This arises from the fact that the ferment cannot ferment maltose. Hansen further established the fact that *Sacch. apiculatus* does not secrete invertase. In dextrose solutions, however, it excites, as Boutroux has shown, vigorous alcoholic fermentation. Hansen found that this ferment gave a fairly active fermentation in 10 to 12 per cent. solutions of dextrose in yeast-water; at the end of the experiment the liquids still contained sugar, a proof that *Sacch. apiculatus* can only carry the fermentation to a certain point.

It was shown by experiments, in which this ferment was cultivated in beer-wort, together with *Sacch. cerevisiæ*, that it, as the weaker, was crowded out by the latter, although it retarded the *Sacch. cerevisiæ* to no slight extent.

In flasks with the same beer-wort, and at the same temperature, and each containing one species, it was found that the *Sacch. apiculatus* had increased to a much greater extent than the *Sacch. cerevisiæ* in the same interval of time.

The ferment may, at the critical time of the year, make its appearance in large quantities in the wort, and exist side by side with the *Sacch. cerevisiæ* and retard its action; when, however, the beer is transferred to the lager-cellar, the ferment remains inactive in the alcoholic liquid and frequently dies,

The most interesting phase in the life of this ferment is its conditions, described by Hansen, in free nature. By microscopical investigations and cultural experiments it was shown that during the summer the ferment occurred in free development on the sweet succulent fruit (cherries, gooseberries, strawberries, grapes, plums, &c.) during their ripening. On the contrary, it was only found quite exceptionally on the same fruit in an unripe state. Whilst it is found in vigorous budding on the above-mentioned ripe fruits, yet it is never, or only very exceptionally, found on other fruits, leaves, twigs, &c., &c.; therefore it was proved that *Sacch. apiculatus* had its peculiar habitat on ripe fruit of that kind. It was also further proved that it always, without exception, was found in the earth under the cherry and plum trees, grape-vines and other plants on whose fruit it occurred, yet only extremely seldom in the numerous samples of earth from other and most diverse localities. Without doubt the fallen fruit and the rain brought the ferment down and into the earth under the trees, and the question then arose whether it wintered there also. The answer was obtained in a double manner: partly by taking numerous samples of the earth from these places during the course of the winter and spring—these, when transferred to wort, gave in the majority of cases a vigorous growth of our ferment—partly by introducing with all care cultures of *Sacch. apiculatus* into the earth, and leaving it to itself during the winter. In the spring and early summer this earth was again examined, and the culture experiments showed that the ferment was alive in all cases. In this way it was proved that the ferment can winter in the earth, as it was previously shown that it can really only be found at the stated places in the ground.

Finally it remained to be proved that the earth is its proper habitat in the winter time; this was done by Hansen investigating from January to June the dust from the most diverse places, also the dried fallen fruit from many plants, and finally also different excrements. These seventy-one analyses gave a negative result, and thus evidence was obtained that the proper winter habitat of the ferment is the earth under the previously mentioned plants. It retains its ordinary appearance during the long winter-time, and is again conveyed into the air during the summer-time by the united force of insects and the wind.

It is clear that the ferment, from the time when it occurs in abundant quantity on the above-mentioned ripe fruit, may be carried by the air currents to other places, and also on to unripe fruit. Even in his first memoir Hansen stated that the unfrequent occurrence on unripe fruit might be due to the premature death of the ferment, partly on account of deficiency of nutriment, and partly on account of the drying up of the cells. Later, this *savant* has experimentally proved the correctness of this supposition. He stirred up with water partly young and partly old cells, and placed them in thin layers either on object-glasses or on thin pulled-out tufts of cotton-wool; after which they were allowed to dry, shaded from the sun. At the end of less than twenty-four hours all the cells were dead. It was self-evident that the isolated cells lying on the unripe fruit are under still more unfavourable conditions than in the experiments. If, however, the cells are spread in thick layers on cotton-wool or filter-paper, they remain, as in the earth, living for a long time; for instance, over eight months in filter-paper.

Mycoderma cerevisiæ and vini (Saccharomyces mycoderma).

These sprouting-fungi still belong, in spite of the endless literature which exists on the subject, to the least known among the organisms of fermenting liquids, since the question of species has not yet been definitely settled. They occur in

cultures very readily, and have caused—according to Bêlohoubek's observations in Bohemian breweries—very serious money losses by their vigorous development in beer.

The Mycoderma (*cerevisiæ*) occurring on beer and wort forms, as is known, a dull, greyish, often matted film. Hansen obtained a similar growth when he exposed Carlsberg lager-beer in open vessels at low temperatures—2° to 15° C. The ferment is developed even at 33° C. ; at temperatures above 15° C., however, it gives place more and more to competing forms, especially when 26° C. is passed. Therefore, as favourable conditions exist at low temperatures for its development, there are very good grounds for dreading its increase in the storage cellar, especially as lager-beer is a much more favourable nutritive liquid than wort for this organism. This is well shown by the following experiment. When Hansen transferred traces of a pure film to lager-beer and wort contained in open vessels, and allowed these to stand in the open air where various germs were present, he found that the culture in beer nearly always remained pure, whilst various additional species were present on the wort.

Close microscopical examinations show that Mycoderma can occur in different forms ; the cells are usually transparent and less refractive than those of the true Saccharomycetes ; in each cell there are generally present one, two, or three very refractive bodies, which often have a vibratory rolling motion.

Winogradsky states that *Mycoderma vini* obtained in pure cultivation by Hansen's method, alters its form with the composition of the nutritive solution ; he experimented partly with solutions, of which the mineral constituents remained constant, whilst the organic substances varied, and partly with solutions, of which the reverse was the case.

Although de Seynes and some other naturalists claim to have found ascospores in this ferment, yet it appears from later work that it is not possible to bring about this formation ; the figures in question show that the fat globules, which occur in many unicellular ferments during the resting stage, had been mistaken for spores. The old name, *Mycoderma*,

is therefore more appropriate for this ferment than the new *Saccharomyces*.

It has also not yet been satisfactorily determined whether *Mycoderma cerevisiæ* and *vini* are different species.

An advance was, however, made when Hansen found a purely macroscopic distinguishing feature for this Genus, or this group of film-forming yeast cells. As we previously mentioned, the different species of *Saccharomyces* and *Saccharomyces apiculatus* give colonies in a mixture of wort and gelatine which cannot be clearly distinguished one from another. It is quite otherwise, however, with the varieties of *Mycoderma*. Whilst the true Saccharomycetes give in gelatine specks of a bright greyish-yellow colour, with dry or lustrous surface, and shaped like small pin-heads, the perforated specks of *Mycoderma cerevisiæ* and *vini*, and some closely related species are, however, bright grey, and spread out like a membrane, or hollowed out like a shell. When this has been seen even once it is very easy to recognize these specks.

With regard to the genetic connection of these ferments with the fungi in general, there exists a very large amount of literature, which shows at the best how few real characteristics there are for the examination and determination of the growth.

These ferments bring about marked alterations in substrata. In this respect we find in the literature of the subject statements that they can excite a feeble alcoholic fermentation when immersed in a saccharine nutritive solution, but Hansen has recently shown that submerged *Mycoderma* is incapable of producing alcohol in saccharine solutions. It is also stated that, when grown on the surface of various liquids, they can bring about an oxidation fermentation, whereby alcohol is converted in some cases into carbonic acid and water, in others, into acetic acid; they may also form fatty acids, then oxidize these and produce ethereal salts (Schulz).

CHAPTER VI.

THE PRACTICAL EMPLOYMENT OF THE RESULTS OF SCIENTIFIC INVESTIGATIONS.

The question: What results, capable of direct practical employment, can be obtained from the facts we have given in the previous chapters?—may be very briefly answered in the present chapter.

Pasteur has proved that bacteria have a very injurious influence on the alcoholic fermentations, and are able to produce diseases in the fermented liquids.

Some advanced technologists, English especially, had carried out microscopical investigations of yeast, proceeding from the same standpoint, before Pasteur published his celebrated work; the honour, however, belongs to Pasteur of having made clear the importance of such investigations to technology.

We therefore work in such a way that contaminations of this kind are prevented, and this is best effected by allowing impure air no entrance to the liquids. For breweries the result of this endeavour has especially been the removal of the open coolers and cooling apparatus, the aëration of the worts by air previously sterilized by heat or filtered through cotton-wool, and the purification of the air in the fermenting rooms. That all these precautions first possessed a real importance through Hansen's system of yeast culture is self-evident.

The statements in Chapter VII. of "Études sur la Bière" regarding the importance of the oxidation of the wort during cooling are also of great value. By direct measurement of the amount of oxygen in the wort, Pasteur showed that a certain amount of oxygen, partly in a free state and partly

in a combined state in the wort, influenced the course of fermentation and brightening, but that when the amount of oxygen in the wort exceeded certain limits it had an injurious influence on the character (*force et arome*) of the beer (p. 377).

One result of Pasteur's work on the bacteria has been, as already stated, a new method of manufacturing vinegar. This method consists in exposing a large surface of the liquid employed, which is composed of two parts of bright wine to one part of vinegar; a young growth of vinegar-bacterium is sown on the surface, and when the temperature and the composition of the liquid are favourable the acidification takes place more rapidly than in the other methods employed in this industry. The installation is said to be cheaper, and the loss of alcohol very little more than in the Orleans process. The method, nevertheless, has been but seldom employed even in France. The cause of this is seen in the fact that the composition of the nutritive liquid is variable, but to a still greater extent in the fact that the growth is impure, and will probably contain species of bacteria with different qualities, different requirements, and giving, of course, different products in varying quantities. This will still be true, even if the growth consists only of those species which are all capable of producing vinegar. In 1879 Hansen has proved to us that under the form of *Mycoderma aceti* are included at least two different species, *Bacterium aceti* and *Bacterium Pasteurianum*. The pure culture of a systematically selected variety of vinegar-bacterium must be, as Hansen has taught us, the starting-point in this industry also.

The method of Appert for preserving fermented liquids was improved by Pasteur, and has been of great importance not only in the manufacture of beer, but also in that of wine and vinegar. In the latter case, however, the honour belongs not to Appert, but to the Swedish chemist Scheele, who employed with success the method of sterilizing vinegar one hundred years ago.

The experiments on aëration described in Pasteur's work mentioned above gave rise to a large series of researches, by

which valuable information on the fermentative and reproductive power of yeast in the presence of varying amounts of oxygen was obtained. These results are, however, of only theoretical interest for the brewer, but in the distillery and pressed-yeast manufactory they play a very important part.

A large number of researches have been made by various authors regarding the behaviour of bacteria in the spirit and pressed-yeast industries; these, however, have all been performed in a very general manner, and therefore lead to no definite conclusions in single isolated cases. This results principally from the fact that there are at present no well-defined distinguishing characteristics for these micro-organisms.

With Hansen's researches on the alcoholic ferments there began, as Aubry aptly says, a new era in the history of the fermentation industries. He has proved by his scientific investigations that there are found, within the limits of the budding-fungi, species whose characteristics can be employed in technological analyses. From this and by experiments on a large scale under the ordinary practical conditions, Hansen has demonstrated that the dreaded yeast-turbidity, and indeed the majority of the commonest and worst diseases of beer are not, as is generally stated in numerous communications to the technical journals, caused by bacteria, by the water, by the malt, by the particular method of brewing, or by something of this kind, but that these diseases must be referred to the yeast itself, since the pitching yeast contains, in addition to the cultivated races, other Saccharomyces which act as disease germs (*Sacch. Pastorianus III.*, *Sacch. ellipsoideus II.*, and *Sacch. Pastorianus I.*), and largely increase during the after-fermentation when the liquid is stored.

Systematic studies, extending over several years, on the constancy of the characters of varieties of brewery yeast have led Hansen to the conclusion that it is fairly easy to bring about for a time very considerable variations in different directions; these, however, will again disappear by suitable culture, and the variety will return to its original condition;

it is, therefore, not possible to produce new varieties. Under the ordinary conditions of the brewery the Saccharomycetes show only very slight fluctuations; these must, therefore, be dealt with in practice as with constant varieties, and the practical methods must be regulated accordingly.

From numerous practical analyses, performed according to Hansen's method given above, also results the lesson that the rules generally given for judging in practice a sound fermentation do not suffice for determining the existence of the above-mentioned disease, since both the head of the liquid as well as the attenuation, breaking and brightening may be perfectly satisfactory, whilst the yeast is still largely contaminated with disease germs. The same remarks also apply to those cases where beer acquires an unpleasant bitter taste; this is likewise caused by disease germs (*Sacch. Pastorianus I.*) in the pitching yeast.

Relative to the forms causing yeast turbidity, Hansen has proved that they may be present in the pitching yeast to the extent of 2·44 per cent. without at any time exerting an injurious influence, provided the process is carefully carried out.

In order to avoid in practice these and other disasters which are produced by wild yeast, Hansen has elaborated :—

1. A method for the practical analysis, by means of which it is possible to guard against the excessive increase of such germs. Researches carried out by Holm and Poulsen have shown that it is possible to detect by this method an admixture of 0·5 per cent. of wild yeast, and, as mentioned above, 2·44 per cent. causes no damage under favourable conditions.

2. A method for the purification of yeast from all foreign organisms, since a cell of the desired cultivated yeast can be separated and grown until a sufficient quantity of absolutely pure yeast is obtained; this can be transferred to the fermenting room and be further propagated there.*

* Hayduck has described an entirely different method for the "regeneration" of bottom-yeast. When he examined yeast after continued use in the brewery, he found that the percentage of nitrogen in the pitching-

These absolutely pure and systematically selected races of yeast, prepared in large quantities for industrial purposes,

yeast increased from one fermentation to another, and in many cases the yeast became at the same time more fermentative ; he concluded therefore that the degeneration of yeast stands in close connection with this, and then endeavoured to make the degenerated yeast poorer in nitrogen. This was effected by growing the yeast in a 10 per cent. cane sugar solution (100 litres of the liquid contained 10 kilos of sugar, 300 grams of hops, and sufficient potassium phosphate and magnesium sulphate). This procedure was introduced into about ten breweries, and Hayduck states that he has generally obtained almost entirely good results.

According to communications from Aubry, yeast analyses have been carried out for a long time in the laboratory of the Scientific Station at Munich, in which special attention was paid to the changes in the amount of nitrogen in the yeast. These have, however, given results, from which it does not appear to be possible to bring the increase and decrease of nitrogen into undoubted connection with the fermentative power. "The fluctuations in the amount of nitrogen in normal yeast in the various successive generations are very great (they may amount to more than 1 per cent.), without marked alterations in the duration of the fermentation and the degree of attenuation being noticed. Further, an increase of nitrogen does not always take place by the repeated use of one and the same yeast in the same brewery ; in fact, according to our observations, this happens comparatively seldom."

Bêlohoubek, of Prague, expresses himself in the same sense : "From my accurate experiments I can also register cases where I was not able to detect even the slightest fault in the beer-yeast itself, where I could determine, even by the employment of the greatest care in its examination with the microscope, no defect in the yeast, and yet it was not possible to obtain favourable results by the employment of this yeast on a large scale. When I had determined in such a yeast the amount of residue, the nitrogen, the ash constituents as well as the phosphoric acid, I very frequently found no other departures or differences from the mean value of these numbers than those which fell between the usual limits and were also accustomed to occur with analyses of other samples of yeast which had behaved most satisfactorily in the fermenting vessels."

It must also be remembered that the method recommended by Hayduck is not—as he himself says—universally applicable, but can only be used in certain cases with success ; therefore such a cultivation of yeast in sugar solutions must at any rate always be regarded as a mere provisional aid and not as a true purification, *i.e.*, a separation of those micro-organisms which bring about injurious alterations in the nature of the product. This is in all cases only possible by Hansen's methods of pure culture.

are now—although it is scarcely three years since the first experiment was made in the well-known brewery of Old Carlsberg, Copenhagen—introduced into numerous breweries in all beer-producing countries.

A very considerable number of pure cultures have been recently sent out from my laboratory, cultures both of top-fermentation as well as bottom-fermentation yeast. Numerous pure cultures of bottom-yeast have also been issued from the Munich and other Scientific Stations, and communications regarding these have been published by the director, Dr. Aubry, by Dr. Will, and later by Professor Lintner. Some of these opinions are appended.

Professor Lintner gives the following *résumé* of the general advantages of the process* :—

"Now that different breweries have employed the Carlsberg pure cultivated races of yeast, and also that the Scientific Station at Munich has introduced pure cultivated yeast from the ordinary Munich yeast into various breweries, we are in a position to make the following generalizations from the results obtained :—

"1. By contamination with so-called wild yeasts, an otherwise normal brewery yeast may be gradually rendered incapable of producing a beer of good flavour, and with good keeping properties.

"2. A contamination with wild yeasts may be produced by the dust of the air during summer and autumn, by the introduction of other yeasts, or by the conditions of storage.

"3. By employing Hansen's method of pure cultivation and analysis, it is possible to obtain from a contaminated yeast a good brewery yeast in a state of purity.

"4. Yeast cultivated in a state of purity possesses in a marked degree the properties of the original yeast before contamination, as far as regards the degree of attenuation, the flavour, and the keeping properties of the beer.

"5. There exist different varieties of normal bottom yeasts (*Sacch. cerevisiæ*), each with special properties, and these distinctive peculiarities of the varieties remain constant."

* "Zeitschrift für das gesammte Brauwesen," 1885, p. 339.

Dr. Aubry says*: "In addition to the breweries already mentioned (Spatenbräu and Leistbräu, Munich), a large number of both home and foreign breweries have obtained pure yeast from Carlsberg, and carried out experimental fermentations with it. Naturally the results which had been expected were not in all cases attained, the degree of attenuation was, in the greater number of cases, found to be too low,† the taste was not the one desired, &c., &c., but all reports which are known to us speak favourably of the soundness and brightness of the beer and its freedom from any taste of yeast. The good properties of the yeast have also caused many breweries to introduce it permanently, as, for example, the Liesinger brewery in Liesing, near Vienna. During the present brewing season the Spaten brewery in Munich has made an extensive use of yeast obtained from Carlsberg, and the pitching yeast used in the brewery of the Franziskanerkeller, in Munich, during the greater part of the winter was also derived from a pure culture from Carlsberg.† The course of fermentation and the results regarding the taste, aroma, and soundness of the beer, answered all requirements. The somewhat lower attenuation appeared to be a characteristic of the yeast, since it remained constant. The taste of the beer is at first the ordinary Munich taste combined with something different; with later generations it approaches more nearly the latter, but remains always soft and good."

Will writes‡: "If now, as I trust to have made clear, there is proved the possibility of detecting with certainty the species of yeast which have an injurious influence in practice, we must show a practical use for this knowledge and only employ such materials as do not show the above-mentioned characteristics for the injurious species which so frequently cause disasters in the brewery in the most marked manner. This, however, will only be possible when yeast cells endowed with the properties of normal bottom yeast are isolated from ordinary brewing yeast and further culti-

* Ibid., 1885.

† Carlsberg Yeast No. 2.

‡ "Allgemeine Brauer- und Hopfenzeitung," 1885.

vated with the exclusion of every contamination; in other words, when only pure cultivated yeast is employed in the brewery. Hansen deserves in this respect the great credit of having pointed out the way and devised a method which enabled him to attain the desired end. The far-reaching results which were produced in Old Carlsberg with the pure cultivated yeast, have already caused many other breweries to work only with pure cultivated yeast, and the results are in general received with satisfaction, if only the varieties are chosen to correspond with the normal bottom yeast as far as the requirements of attenuation and taste are concerned.

"May therefore the knowledge of the value of the pure cultivated yeast penetrate in ever-widening circles, and thus many old prejudices concerning yeast be dispersed; may, however, the smaller breweries, which without this have to contend with many difficulties, not stop with the conviction that with the introduction of pure cultivated yeast into an otherwise well-conducted brewery a series of calamities must be overcome. The amount laid out will bring a rich interest."

Louis Marx, of Marseilles, speaks in a similar manner.

One of the first important results which was seen when Hansen's measures of reform had been practically tested in various places, was that there was really concealed under the special name *Saccharomyces cerevisiæ* a series of species or varieties which exhibited the most diverse properties. There was, therefore, a wide prospect opened in this for the determination and domination of the course and the results of the fermentation, and even now important practical knowledge has been obtained.

It is shown by numerous experiments that the characteristics which belong to the different species or races are so strong and so much a part of the cell, that they appear in the same manner in all important particulars when the same variety is employed in breweries under very different conditions. As an example of this may be mentioned the so-called Carlsberg variety No. 1, which always gives beer with a very great resistance to yeast turbidity. Other remarkable differences in varieties of yeast employed technically

are their power of giving a quicker or slower brightening, a more or less lasting head on beer, a dense or loose sediment in the bottom of the fermenting vessel, a higher or lower degree of attenuation, a greater or less power of withstanding contamination of different kinds—all properties which have long been well known in practice, but which now for the first time, as a consequence of Hansen's discoveries, have been recognized in the proper relation to the character of the species and the purity of the growth. A considerably greater certainty and constancy has been introduced into the brewery in consequence of this.

Since with the different nature of the product, very dissimilar requirements are needed for the course of fermentation and for the final condition of the beer, it follows, as Dr. Jacobsen has already pointed out, that the same variety of yeast cannot be everywhere employed. It is, however, possible, in the manner already shown, to find by careful experiment the variety which serves the best for the given conditions. When this is found the work then only consists in introducing a pure culture of the same yeast into the brewery, whenever it is found that the yeast then in use has degenerated.

The numerous experiments with absolutely pure top yeast, which have been carried out for the first time in my laboratory, have shown that the discoveries of Hansen have great importance for brewing in this direction also. The beer prepared with the pure cultivated top-fermentation yeast has a pure sweet taste, and a much greater resistance to contamination than ordinary beer.

Naturally, great care must be taken in this case also that the culture in the brewery is prepared under conditions of extreme purity.

The production of light, top-fermentation, and thoroughly sound beers, poor in alcohol, has certainly a future in many beer-brewing countries, especially when the product is of better quality than formerly during the warmer months of the year. An important condition for this is, according to the collective experience, a pure pitching yeast, and it will certainly be better for Bohemia, as Bělohoubek has also

pointed out, when the top-fermentation breweries, instead of working partly for bottom fermentation, introduce improvements in their top-fermentation processes in accordance with the spirit of the age, and thereby bring about a real improvement in this product; as producers of bottom-fermentation beer such breweries could in any case compete with the already established large concerns.

The question, How long does a pure culture remain in its original good condition?—cannot at present, and perhaps, also, not in the future, be answered with certainty. As already mentioned, the varieties possess a different resistive power against contamination; my connection with different breweries has also shown—which might have been expected—that the same variety does not remain pure and good for the same length of time in the dissimilar fermenting rooms where it was employed. We know that the time of year also plays a part here, and that the period of the year when the yeast forms are found in the largest quantity in the air is especially dangerous. As is known, contamination may take place at other times of the year, especially from utensils and plant generally. It is, therefore, little probable that this question will find an answer generally applicable to the practice. This is, however, of less importance when we remember that an analysis will always show the infection long before it becomes dangerous, and that a new pure culture of the same yeast can be at once introduced. The principal result is that it is no longer necessary to work at hazard, and it is not now necessary, as formerly, to leave the fermentation to chance.

At the present time, when this English edition appears, Hansen's system has been introduced with complete success in numberless breweries, not only in Europe, but also in America, in Asia, and in Australia, and his discoveries now promise to bring about a reform in the spirit and pressed-yeast industries, as well as in the manufactures of wine and vinegar.

APPENDIX.

THE PREPARATION OF PURE YEAST ON AN INDUSTRIAL SCALE.

IN the foregoing pages the methods for the preparation of a pure culture of yeast from a single selected cell are fully described, but the matter is carried no further than the introduction of the yeast colony into a small Pasteur's flask containing nutritive liquid. For the English reader, however, it appeared advisable that some description should be given of the means adopted for preparing larger masses of pure yeast for brewery purposes, and this Appendix has been written with that object in view.

In Hansen's earlier experiments in this direction he employed four or five ordinary Pasteur's flasks (Fig. 3) of about 1·25 litre capacity, and four metallic vessels, each holding about 10 litres. These vessels are shown in Fig. 37; they are constructed of tinned copper, and are on the principle of a Pasteur's flask. The free ends of the short tubes *a* and *b* are inserted in pieces of india-rubber tubing, which are closed at the other end with short pieces of glass rod; in addition, the india-rubber at *b* is closed with a screw-clip. The bent tube which proceeds from the top of the vessel is jointed with india-rubber tubing at *c*, and the lower end of the tube is closed by a cap of cotton-wool, *d*; at *e*, the tube is enlarged in order to guard against contamination after sterilization. The nutritive solution employed in the Pasteur's flasks and in these vessels is ordinary hopped wort of about 1056 gravity, and it is thoroughly sterilized by boiling with all proper precautions. More especially in the case of the metal vessels it is necessary that some months should elapse

between the sterilization and use of the vessels, in order that the wort may become thoroughly aërated, as it has been shown by Hansen, Aubry, and Jörgensen that a selected variety of

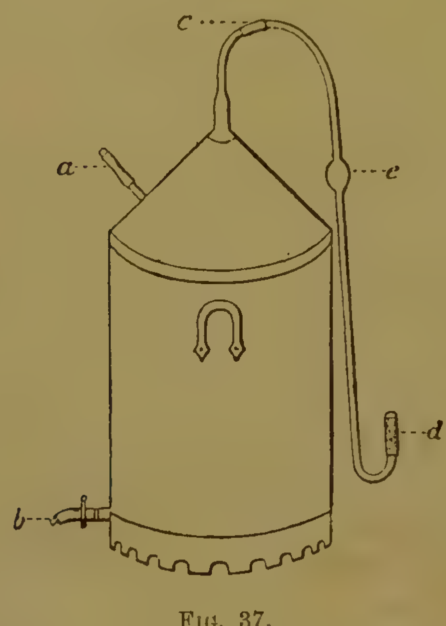


Fig. 37.

yeast does not give a normal fermentation unless the wort employed for its cultivation is well aërated.

The manner of preparing a cultivation in these vessels is briefly as follows:—The colony of yeast is selected in the manner described in a former chapter, and then transferred to one of the small $\frac{1}{4}$-litre Pasteur's flasks. When the fermentation in this flask is at an end, the fermented liquid is poured off, and the sediment divided between the four or five large flasks containing beer-wort. Four only of these are required; the fifth serves as a reserve in case of accidents. Fermentation is now allowed to proceed at the ordinary room temperature, and at the end of about seven days a considerable sediment of yeast has formed. The greater portion of the fermented liquid is poured off, only sufficient to render the yeast fluid being allowed to remain. The sediment from each

flask is now added to one of the metal vessels (which should contain from 7—8 litres of beer wort) through the tube *a*, Fig. 37. Vigorous fermentation of course takes place, and at the end of seven days the four vessels together contain sufficient yeast to barm about 1 hectolitre of wort. This amount

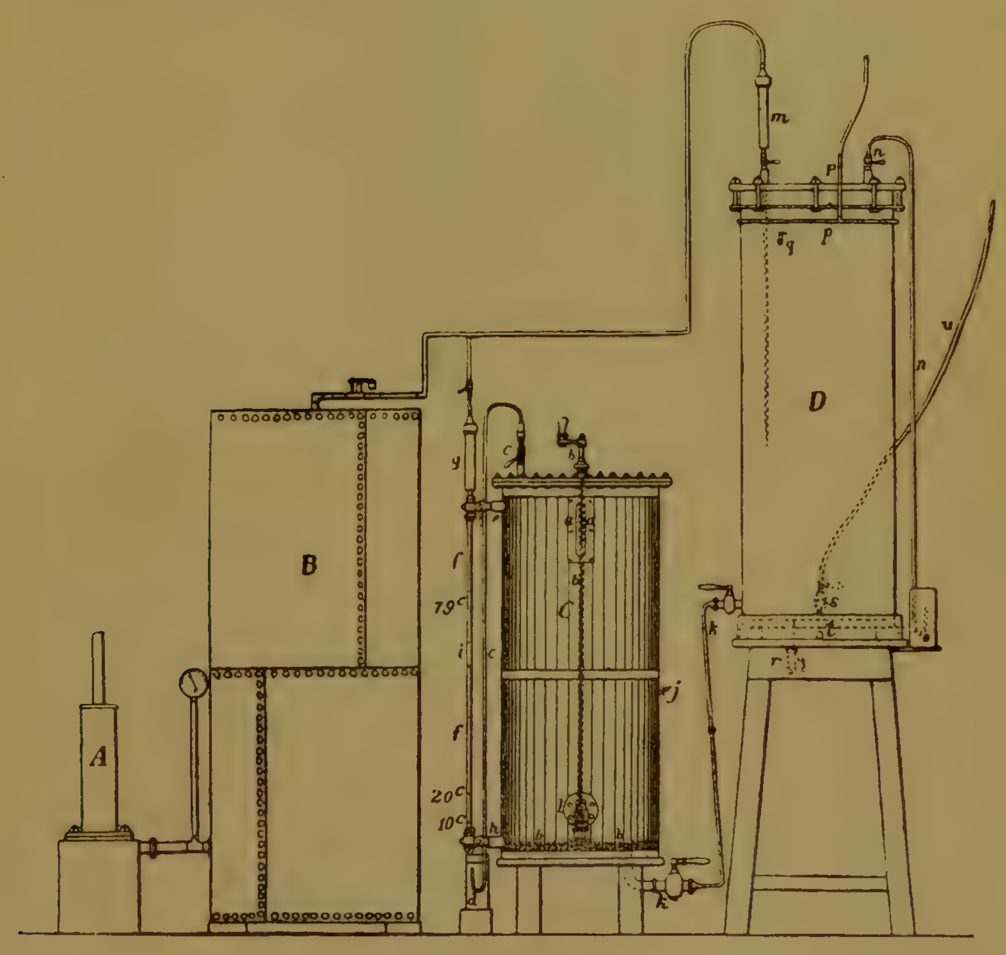


Fig. 38.

of yeast can be transferred to a small fermenting vessel in the brewery, and gradually increased until a sufficient supply is obtained for any quantity of wort.

It is evident that up to the point at which the yeast leaves the metal vessels all outside contamination is rendered

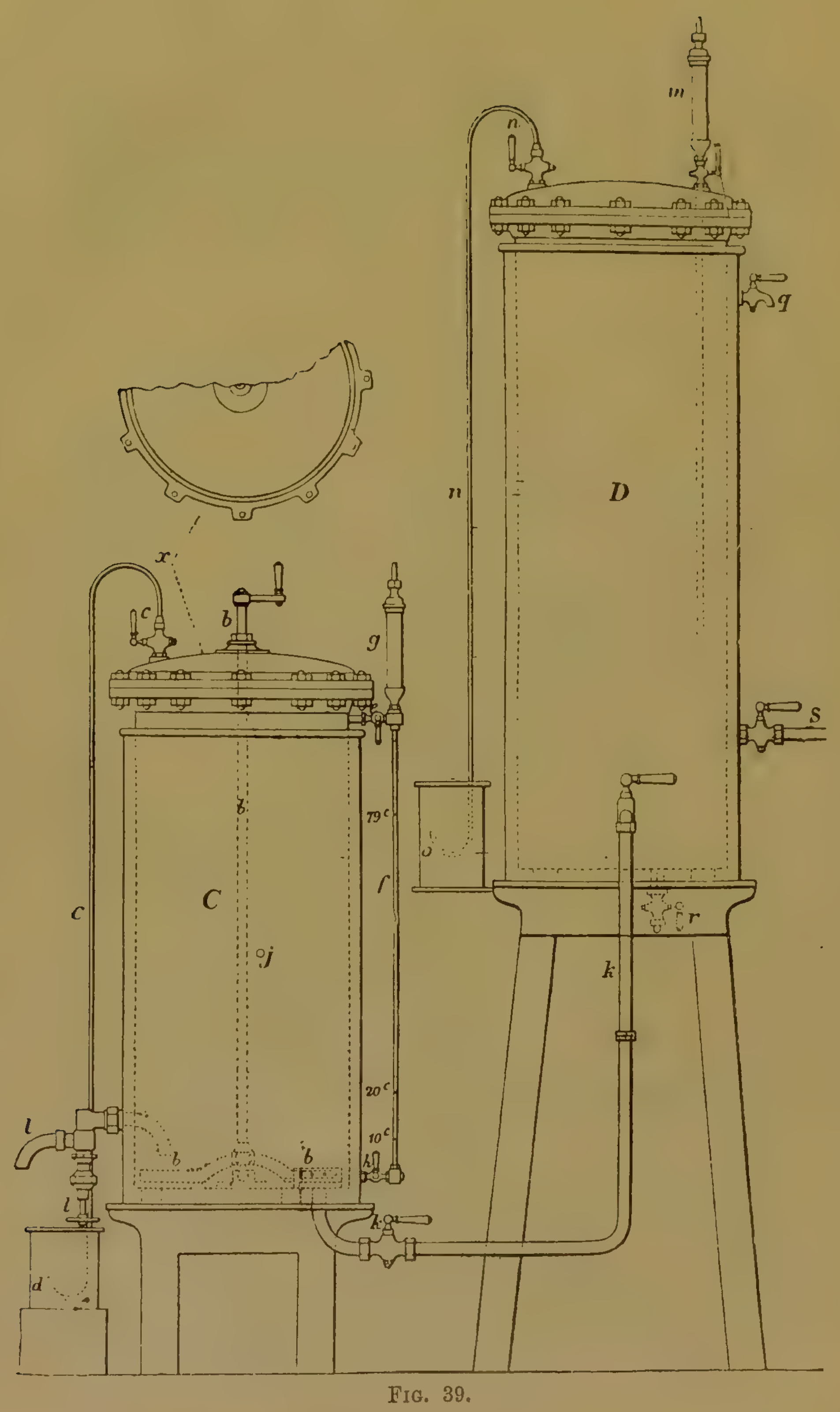


FIG. 39.

impossible, provided the necessary precautions are taken in transferring the yeast from one vessel to another. This is a matter which requires considerable experience and a suitable working place, the details of which have been earlier described.

In order to avoid the slight amount of uncertainty connected with the preceding method, Hansen has devised, with the aid of Kühle, an apparatus which enables him to secure a constant supply of the same variety of yeast, and thus to introduce, if necessary, a fresh cultivation of the yeast into the brewery every seven days. This apparatus is shown in the appended Figures 38 and 39. Fig. 38 shows the entire apparatus, consisting of three principal parts with the connecting tubes, namely—

1. The arrangement for aërating the wort, consisting of an air-pump (*A*) and air-reservoir (*B*);
2. The fermenting vessel (*C*); and
3. The wort-cylinder (*D*).

The latter two parts are shown more in detail, and with the casing of *C* removed, in Fig. 39.

The apparatus is very simple in construction, and the end aimed at throughout is to collect wort in a sterile state, to introduce yeast and allow the fermentation to take place in the absence of all aërial contamination, and to remove the fermented liquor and yeast without contaminating the interior of the vessel. The following brief description will suffice to explain the construction of the apparatus and the manner of working it:—

The air-pump, *A*, is working by an engine, and supplies the air-reservoir, *B*, with air compressed to about one to four atmospheres. The air is purified by filtration before entering the reservoir, and again before entering the wort-cylinder, *D*, which is previously sterilized by super-heated steam; it is of course most essential that every part of the apparatus—all pipes, taps, &c.—should be thoroughly sterilized before commencing operations. This is done by super-heated steam and boiling water. The tap, *s*, is connected with the main supply from the copper, and the wort-cylinder filled with boiling hopped wort; cooling is effected by means of a stream of cold

water, which circulates in the hollow jacket, and the necessary amount of air for aëration of the wort is allowed to enter through the filter, *m*, the inner tube of which dips some little distance into the wort, as shown by the dotted lines. The fermenting vessel, *C*, is sterilized in the same manner as the wort-cylinder, the steam passing into the vessel through the filter, *g*, which also subsequently serves to filter air ; it is provided with an exit tube for the carbonic acid, *c* ; a stirring apparatus for mixing the wort and yeast, *b*, *b*, *b*, *b* ; and a small tube through which the yeast is added, and also small samples of the fermenting liquid can be removed, *j* ; the tap, *l*, for drawing off the fermented liquid is constructed in such a way that the liquid itself provides the means of purification, and prevents any contamination from without taking place at this point.

The wort-cylinder must be placed at a higher level than the fermenting vessel, in order to allow the wort to flow from one vessel to the other through the connecting tube, *k*.

When it is intended to instal a culture of yeast in this apparatus, the cylinder, *D*, is filled with wort, and this allowed to run into the fermenting vessel until it is a little below the level of the tube, *j* ; the small growth of yeast from the laboratory is added through this tube, and then the wort again allowed to run in until 220 litres are present. Fermentation now takes place, and is complete at the end of about ten days ; the greater part of the beer is now run off through *l*, sterilized air being meanwhile allowed to enter through *g* and *c* ; a little fresh wort is now run in from *D*, and well mixed with the yeast by means of the stirrer, *b*, *b* ; 27 litres of the mixture are withdrawn, and the process repeated, a second 27 litres being thus obtained. In this way sufficient yeast is obtained in the 54 litres of the mixture to barm 8 hectolitres of wort. The fermenting cylinder is again filled up to 220 litres, and the amount of yeast remaining in the vessel is sufficient to again start the fermentation. This process may be repeated every ten days, and thus one fermenting cylinder will yield sufficient absolutely pure yeast to barm 24 hectolitres of wort each month.

Hansen states that the two chief points to be borne in mind in working with this apparatus are thorough and complete sterilization by super-heated steam, and the presence of an excess of sterilized air in the respective cylinders during the cooling and withdrawal of the wort and fermented liquid.

This apparatus is at work in very many breweries on the Continent, in the United States, and elsewhere, whilst modified forms are being worked at Rotterdam by Dr. Elion, at Marseilles by Louis Marx, and at Burton-on-Trent in the brewery with which the writer is connected.

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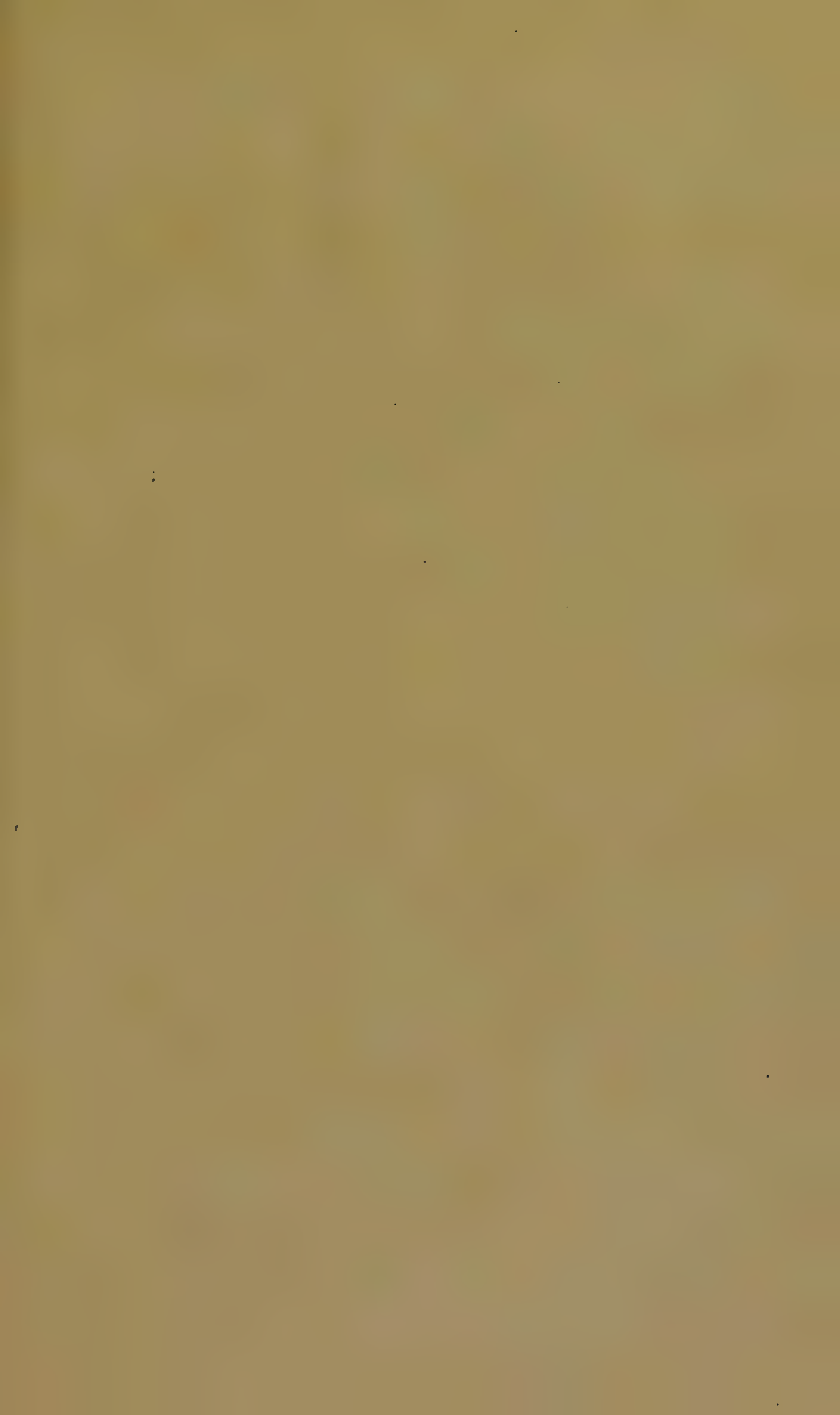
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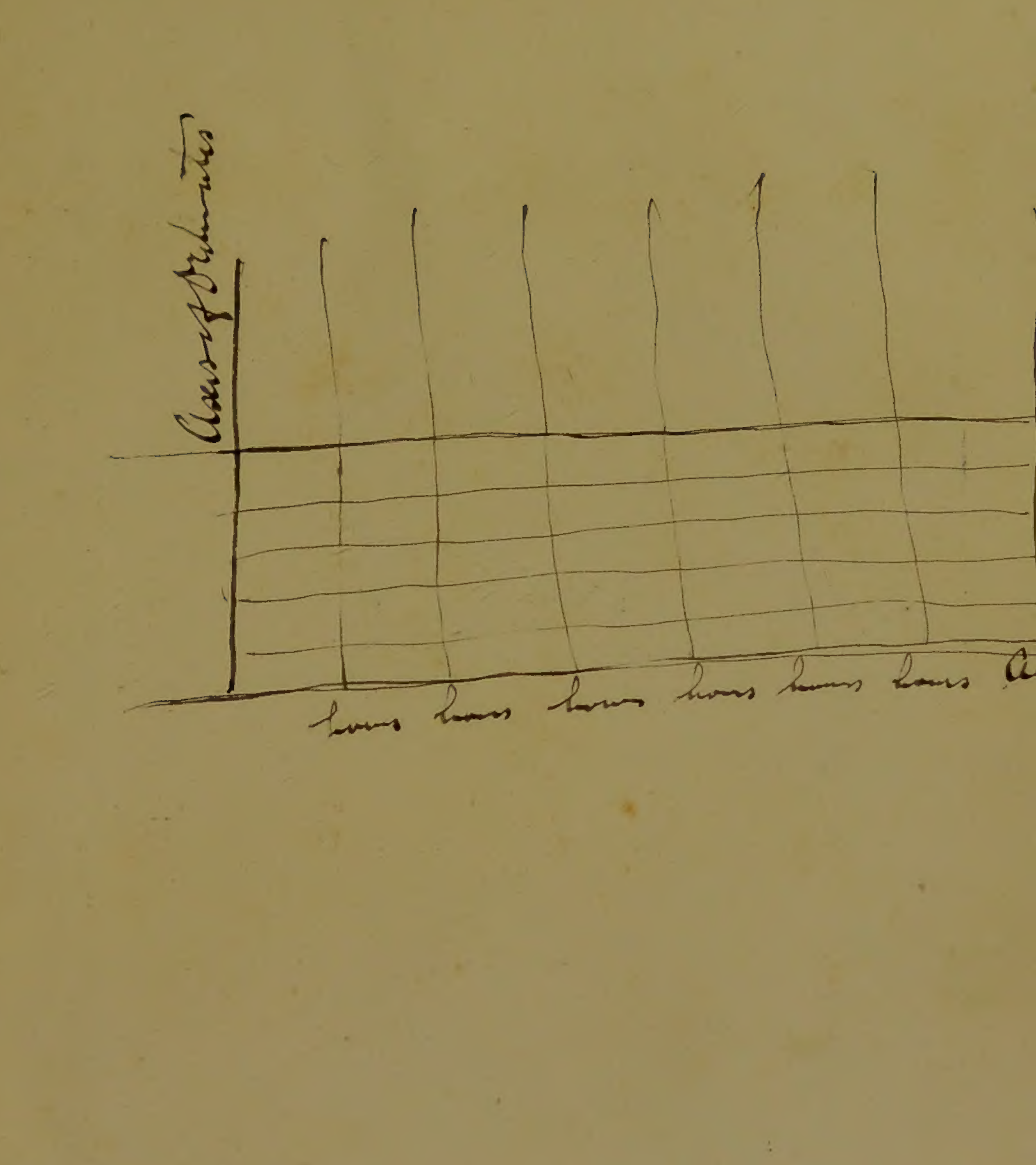
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